REMARKS

Claims 1-20 and 58-99 are pending and under examination. Claim 21-57 were previously canceled. Claims 1-20 and 58-79 have been canceled without prejudice to Applicants pursuing these claims in a related application. Claim 80 has been amended to identify in the preamble and in the body of the claim the method is directed to differentiating progenitor cells "*in vitro*." In particular, support for amendments to claim 80 can be found on page 59, lines 9-11. Accordingly, the amendments to do not introduce new matter and entry thereof is respectfully requested. Entry of the proposed amendments is respectfully submitted to be proper because the amendments are believed to place the claims in condition for allowance.

Upon entry of the above amendments, claims 80-99 are currently pending.

For the following reasons, this application should be considered in condition for allowance and the case passed to issue.

Priority

Applicants thank the Office for acknowledging Applicants' claim for domestic priority to the Provisional Application Serial Number 60/209,539, filed June 5, 2000 (herein "the provisional application"). However, the Office asserts that this provisional application allegedly fails to provide adequate support under 35 U.S.C. §112 for claims 1-20 and 58-99 for the same reasons as applied to the present application. Applicants respectfully traverse for the same reasons of record and further direct the Office to the arguments presented below with respect to the currently pending claims.

Claim Rejections – 35 U.S.C. §112, First Paragraph - Enablement

The rejection of claims 1-20, 58-79 and 80-99 under 35 U.S.C. §112, first paragraph for lacking enablement, is respectfully traversed. Although Applicants maintain that the claims are enabled as previously presented, in an effort to further prosecution, claims 1-20 and 58-79 are cancelled herein rendering the rejection of these claims moot. Applicants herein will address the above rejections as they apply to pending claims 80-99.

The Office states that the specification is enabling for a method of differentiating progenitor cells *in vitro*, comprising the steps of (a) contacting *in vitro* said progenitor cells with retinoic acid and (b) introducing *in vitro* into said progenitor cells a nucleic acid molecule encoding an MEF2C polypeptide, wherein said progenitor cell is selected from the group consisting of a P19 and a mouse embryonic stem cell, thereby differentiating said progenitor cells to produce a cell population containing neuronal cells protected from apoptotic cell death, but allegedly does not provide enablement for the full scope of the claims. The Office cites the eight factors of *In re Wands*, 858 F. 2d 731 (Fed. Cir. 1988).

Applicants submit that the specification fully satisfies the requirement for enablement under 35 U.S.C. §112, first paragraph for the claimed methods. "The law does not require a specification to be a blueprint in order to satisfy the enablement requirement," *Staehelin v. Secher*, 24 U.S.P.Q. 2d 11513, 1516 (Bd. Pat. App. & Int. 1992). A specification need not describe—and best omits—that which is well known in the art. See, e.g., *In re Buchner*, 929 F.2d 660, 661, 18 U.S.P.Q.2d 1331, 1332 (Fed. Cir. 1991). It is also well-settled in the law that "a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." *Ex parte Jackson*, 217 U.S.P.Q. 804, 807 (Bd. App. 1982). Furthermore, enablement does not require absolute predictability. Rather, it requires that a person skilled in the art be able to practice the invention without undue experimentation. *In re Wands,* at 737 & 738. The enablement requirement is met if a preponderance of the evidence indicates that it is more likely than not that any person skilled in the art at the time the application was filed could have practiced the claimed methods.

Claim 80, and all dependent claims thereof, are directed to methods of differentiating progenitor cells *in vitro* to produce a cell population containing neuronal cells protected from apoptotic cell death by: (a) contacting *in vitro* said progenitor cells with a differentiating agent; and (b) introducing *in vitro* into said progenitor cells a nucleic acid molecule encoding a constitutively active MEF2 polypeptide or an active fragment thereof, thereby differentiating said progenitor cells *in vitro* to produce a cell population containing neuronal cells protected from apoptotic cell death.

As taught by the specification, a differentiating agent means a naturally occurring or synthetic cytokine, growth factor or other compound that causes or enhances a progenitor cell to have one or more characteristics of a neuronal cell (page 46, lines 18-31 of the subject application and page 24, lines 1-18 of the provisional application). Examples of such differentiating agents include, but are not limited to, retinoic acid, neurotrophic factor 3, epidermal growth factor, insulin-like growth factor 1 and platelet-derived growth factor, as recited in claims 96 and 97. Progenitor cells include, but are not limited to, stem cells, which can be for example embryonic stem cells such as human embryonic stem cells or, for example, human hematopoietic progenitor cells including the most undifferentiated, and pluripotent hematopoietic progenitor cells, which can be denoted hematopoietic stem cells (page 5, lines 1-7 and page 29, line 7 to page 31, line 9 of the subject application and page 12, line 14 to page 14, line 15 of the provisional application), as recited in claims 85-89. Alternatively, progenitor cells can be positive and/or negative for one or more specific marker, such as for example CD133, CD34, CD38, CD45 Lin and Thy-1 (page 28, lines 8-19 of the subject application), as recited in claims 90-95. Methods for contacting the progenitor cells *in vitro* with a differentiating agent are described in Example 1 of the subject application and in the provisional application.

Additionally, as taught by the specification, a variety of methods are known in the art for introducing a nucleic acid into progenitor cells *in vitro* (page 54, line 4 to page 55, line 17 of the subject application and page 27, line 18 to page 28, line 19 of the provisional application). Such methods include, but are not limited to, microinjection, electroporation, lipofection, calcium-phosphate mediated transfection, DEAE-Dextran-mediated transfection, polybrene- or polylysine-mediated transfection, and conjugation to an antibody, gramacidin S, artificial viral envelopes or other intracellular carriers such as TAT. The specification teaches specific references for many of the methods. The specification states on page 54, lines 15-30:

> For example, embryonic stem cells can be transformed by microinjection as described in Cibelli et al., Nat. Biotech. 16:642-646 (1998) or Lamb and Gearhart, Cur. Opin. Gen. Dev. 5:342-348 (1995); by lipofection as described in Choi (U.S. Pat #. 6,069,010) or Lamb and Gearhart, Cur. Opin. Gen. Dev. 5:342-348 (1995); by electroporation as described in Current Protocols in Molecular Biology, John Wiley and Sons, pp 9.16.4-9.16.11 (2000) or Cibelli et al., Nat. Biotech. 16:642-646 (1998); or by fusion with yeast spheroplasts Lamb and Gearhart, Cur. Opin. Gen. Dev. 5:342-348 (1995). A MEF2 polypeptide also can be delivered to stem

> or progenitor cells as a TAT/MEF2 polypeptide fusion by techniques well known in the art as described in Nagahara et al., Nature Medicine 4:1449-1452 (1998).

Furthermore, the specification teaches that viral vectors can be particularly useful for introducing a nucleic acid molecule in the methods of the invention. Such vectors include, for example, retroviral vectors, lentiviral vectors, adenoviral vectors and adeno-associated vectors (AAV), herpesvirus vectors as described in text books Kaplitt and Loewy, Viral Vectors: Gene Therapy and Neuroscience Applications Academic Press, San Diego, California (1995) and Chang, Somatic Gene Therapy CRC Press, Boca Raton, Florida (1995) (see page 55, lines 1-10 of the subject application). Therefore, the present specification teaches detailed methods for contacting *in vitro* progenitor cells with a differentiating agent and introducing *in vitro* into the progenitor cells a nucleic acid molecule encoding a constitutively active MEF2 polypeptide or an active fragment thereof.

The Office alleges that the specification fails to provide an enabling disclosure for the therapeutic use of the cell compositions produced from the claimed method because at the time the invention was made, successful implementation of cell therapy and gene therapy protocols was allegedly not routinely achievable by those skilled in the art. The Office simply cites the same references of record including: Rossi and Cattaneo, *Nat. Rev. Neurosci.* 3:401-409 (2002); Cao et al., *J. Neurosci. Res.* 68:501-510 (2002); Mehler et al., *Arch. Neurol.* 56:780-784 (1999); Jackowski, *Br. J. Neurosurg.* 9:303-317 (1995); Grados-Munro et al., *J. Neurosci. Res.* 74:479-485 (2003); Filbin, *Nat. Rev.* 4:1-11 (2003); Cheng et al., *Blood* 92:83-92 (1998); Hanazono et al., *Stem Cells* 19:12-23 (2001); and Zwaka and Thomson, *Nat. Biotechnol.* 21:319-321 (2003) and provides the same arguments of record to support the Office's position. The Office also reiterates the same arguments against references of record relied upon by Applicants to support their position including Eiges et al., *Curr. Biol.* 11:514-518 (2001); Ferrari et al., *Gene Therapy* 4:1100-1106 (1997); Uyttersprot et al., *Mol. Cell. Endocrin.* 142:35-39 (1998); Milward et al., *J. Neurosci. Res.* 50:862-871 (1997); McDonald et al., *Nat. Med.* 5:1410-1412 (1999); and Liu et al., *Proc. Natl. Acad. Sci. USA* 97:6126-6131 (2000). Applicants respectfully traverse the characterization of the above references for the reasons of record and further assert the following arguments.

Applicants respectfully point out that although the Office Action states on page 2 that the amendment and remarks filed with the response of August 29, 2008 have been entered, it appears that the Office has not fully considered the arguments provided in the response of August 29, 2008 because the Office Action repeatedly, on pages 10 through 19, refers to the incorrect pages of the response filed August 29, 2008 and specifically on page 16, last paragraph, the Office action refers to comments regarding Example 6, which were part of the response filed July 19, 2007. Furthermore, it appears that the office has not considered the Rule 132 Declaration executed by Dr. Stuart Lipton submitted with the response of August 29, 2008 because the Office Action on page 19 only refers to the Declaration submitted on July 19, 2007 and does not acknowledge or respond to the data provided in Exhibit B regarding transfection of human embryonic stem cells with a lenti-MEF2CA construct provided in Dr. Lipton's Declaration of August 29, 2008.

The Office states that the specification is enabling for contacting *in vitro* said progenitor cells with retinoic acid. Retinoic acid as described in the specification is a differentiating agent, which is defined as a naturally occurring or synthetic cytokine, growth factor or other compound that causes or enhances a progenitor cell to have one or more characteristics of a neuronal cell (see page 46, lines 18-22 of the subject application). The specification also teaches other differentiating agents to include neurotrophic factor 3, epidermal growth factor, insulin-like growth factor 1 and platelet-derived growth factor (see page 46, line 22-27 of the subject application). The Office Action on page 10 states:

> …much work has been done to develop techniques for the directed differentiation of ES cells *in vitro* to produce desired cell types…

As such, Applicants submit that one of ordinary skill in the art would know methods for contacting *in vitro* progenitor cells with a differentiating agent as set forth in the claimed methods.

Applicants submit that the following references do not apply to the claimed methods of introducing *in vitro* into progenitor cells a nucleic acid molecule because each of the arguments the Office presented, which the references allegedly supported, related to enablement of methods introducing *in vivo*: Rossi and Cattaneo; Cao et al.; Mehler et al.; and Cheng et al.

Regarding Jackowski, Grados-Munro et al. and Filbin, the Office Action states on page 14 that the Applicants provide no support for the assertion that these references are not relevant. Applicants respectfully direct the Office to response filed August 29, 2008 bridging pages 10-11, which states:

> In particular, Jackowski is a review article that discusses neural injury repair and "outlines possible therapeutic approaches that may enable more effective CNS regeneration to be accomplished in the future" (abstract; emphasis added). Similarly, Applicants respectfully submit that both Grados-Munro et al. and Filbin are, at best, review articles describing myelin-associated inhibitors of axon regeneration.

Applicants submit that for the above reasons and the reasons of record that Jackowski, Grados-Munro et al. and Filbin are not relevant to the claims methods which are directed towards methods of differentiating progenitor cells *in vitro* to produce a cell population containing neuronal cells protected from apoptotic cell death by: (a) contacting *in vitro* said progenitor cells with a differentiating agent; and (b) introducing *in vitro* into said progenitor cells a nucleic acid molecule encoding a constitutively active MEF2 polypeptide or an active fragment thereof, thereby differentiating said progenitor cells *in vitro* to produce a cell population containing neuronal cells protected from apoptotic cell death.

Regarding Hanazono et al. and Zwaka and Thomson, the Office Action asserts on page 9 and 10 that the art acknowledged that gene transfer into human hematopoietic stem cells was allegedly problematic. The Office Action further asserts in the paragraph bridging pages 14 and 15 "...given the state of the art in June 2000 was such that no one had successfully transfected human ES cells, it cannot be said that methods for transfecting human ES cells was well known in the art." Applicants respectfully disagree with these assertions. Applicants reiterate the arguments of record regarding Hanazono et al. and Zwaka and Thomson with respect to their disclosure of introducing *in vitro* nucleic acids into human hematopoietic stem cells, particularly in view of the disclosures of Eiges et al., Ferrari et al. and Uyttersprot et al. As stated on the record, Hanazono et al. describes the use of retroviral vectors and lentiviral vectors for gene transfer into human hematopoietic stem cells (page 15, right column, to page 16, left column) and Eiges et al. describes the successful transfection of human ES cells using routine methods well known to those skilled in the art at the time of filing of the priority application.

Applicants also submit the disclosure of van Hennik et al., *Blood* 92:4013-4022 (1998), enclosed as Exhibit A. Van Hennik et al. shows the highly efficient transduction of human umbilical cord blood stem cells with a green fluorescent protein gene using a retroviral vector (see Abstract and page 4016, left column). Van Hennik et al. describes that it is common in the art to transduce human hematopoietic cells with murine retroviruses based on the Moloney murine leukemia virus (MoMLV) (see page 4014, left column, lines 10-12). Van Hennik et al. also describe that mice engrafted with transduced cells showed the *in vivo* repopulating ability of the cells was retained (see Abstract and pages 4016-4018).

Applicants further direct the Examiner to the Rule 132 Declaration of Dr. Stuart Lipton, submitted August 29, 2008, which includes data regarding transfection of human embryonic stem cells with MEF2CA. Exhibit B of Dr. Lipton's Declaration provides date showing the neurogenic activity of MEF2C in human embryonic stem cells. See specifically Exhibit 5, Figure 5B which shows representative pictures from lentivirus infected cells. The arrows in Figure 5B represent MEF2CA-induced neurons.

Applicants still further direct the Office to the specification on page 54, line 4 to page 55, line 17, which teaches a variety of methods are known in the art for introducing *in vitro* a nucleic acid into progenitor cells including, but not limited to, microinjection, electroporation, lipofection, calcium- phosphate mediated transfection, DEAE-Dextran-mediated transfection, polybrene- or polylysine-mediated transfection, and conjugation to an antibody, gramacidin S, artificial viral envelopes or other intracellular carriers such as TAT (see page 54, lines 15-30). The specification also teaches that a variety of viral vectors can be used for introducing a nucleic acid into progenitor cells, which are routinely practiced *in vitro*, such as retroviral vectors, lentiviral vectors, adenoviral vectors and adeno-associated vectors (AAV), herpesvirus vectors (see page 55, lines 1-10).

Applicants respectfully maintain that, not only does Hanazono et al. support Applicants' position that viral vectors were routinely used for gene transfer into hematopoietic stem cells, but van Hennik et al. as well corroborates the routine use of viral vectors to transduce human hematopoietic stem cells *in vitro*. Furthermore, Applicants submit that the evidence provided in the Rule 132 Declaration of Dr. Stuart Lipton submitted with the response of August 29, 2008

corroborates Applicants' position that the specification provides sufficient description and guidance to enable the claimed methods. Thus, Applicants submit that it would have been routine for one skilled in the art to use various known *in vitro* transfection methods to successfully transfect human ES cells.

The Office Action on page 5 asserts that the specification does not offer specific guidance as to how the cell compositions produced can be used therapeutically for any given disorder. Applicants respectfully disagree with this assertion. The subject specification and the state of the prior art at the time the application was filed indicate that such methods are well known. The subject application on page 58, lines 17-24 and the provisional application on page 30, line 30 to page 31, line 5 states:

> Cells can be transplanted into a patient, for example, into the brain or spinal cord using well known methods for transplanting or "grafting" neurons as described, for example, in McDonald et al., Nat. Med. 5:1410-1412 (1999), and summarized in Dunnett et al., Brit. Med. Bulletin 53:757-776 (1997). Methods for preventing or ameliorating rejection, for example, using cyclosporinA treatment, also are known in the art.

The Office Action on page 19 concedes:

> [i]t is accepted that the transplanted MEF2CA-ES-derived neural stem cells survive, migrate and differentiate into neurons in the ischemic mouse cerebral cortex [in which] the cell preparation steps were carried out entirely *in vitro*…

Applicants submit that at the time the application was filed, it was routine for a person skilled in the art to transplant or "graft" the product of the claimed methods, specifically a population of differentiated progenitor cells containing neuronal cells protected from apoptotic cell death.

The Office also asserts on page 17, that given that the specification makes it clear that the protected neuronal cells are being produced for use in transplantation protocols to treat a variety of diseases, it is allegedly not evident that low cell numbers would be sufficient to treat even a single patient. Applicants respectfully disagree with this assertion and reiterate the arguments of record. However, regardless of the transfection efficiency, the proper standard for enablement is that the specification teach those of ordinary skill in the art how to make and use the invention without "undue experimentation." MPEP § 2164.01. As held by the Federal Circuit, "providing proof sufficient to justify conducting in vivo procedures on humans, while useful, is not a test of

patentability." *PharmaStem Therapeutics, Inc. v. Viacell, Inc.*, 2007 WL 1964863 at *20 (Fed. Cir. 2007). See also, *In re Brana*, 51 F.3d 1560, 1568 (Fed. Cir. 1995) (holding that applicants need not produce data that could only be obtained from Phase II clinical trials to satisfy § 112, first paragraph). See also, *Ex parte Balzarini*, 21 USPQ2d 1892 (Bd. Pat. App. & Inter. 1991) (human clinical data is not required to demonstrate the utility of the claimed invention, even though those skilled in the art might not accept other evidence to establish the efficacy of the claimed therapeutic compositions and the operativeness of the claimed methods of treating humans).

Furthermore, Applicants submit that the specification contains a teaching of the manner and process of making and using the invention, as described in detail above, and that the Office has provided no reason to doubt the objective truth of the statements contained therein. See *Rasmusson v. SmithKline Beecham Corp.*, 75 U.S.P.Q.2d 1297, 413 F.3d 1318 (CAFC 2005), where the court states a specification disclosure which contains a teaching of the manner and process of making and using the invention ...must be taken as in compliance with the enabling requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

In view of the foregoing arguments and for the reasons of record, Applicants maintain that, in light of the teaching in the specification, what was well known in the art and the corroborative evidence of record, the specification provides sufficient teaching and guidance to enable the claimed methods. Accordingly, Applicant respectfully request withdrawal of this rejection.

Claim Rejections – 35 U.S.C. §102

The rejection of claims 1-4, 18, 58-62, 76 and 79-99 under 35 U.S.C. § 102(a) as allegedly anticipated by Okamoto et al., *Proc. Natl. Acad. Sci. USA* 97:7561-7566 (2000), is respectfully traversed.

Applicants respectfully maintain that the claimed methods are novel over Okamoto et al. Applicants further point out that the priority application, serial No. 60/209,539, was filed June 5, 2000, which provides an enabling disclosure for the claimed methods as described above. The

Office states on page 21 that the rejection stands or falls with the enablement rejection. Applicants respectfully submit that the priority date of the subject application is prior to the publication date of Okamoto et al. based on the arguments of record and the enablement arguments presented above. Therefore, Applicants respectfully submit that Okamoto et al. is not proper prior art. Accordingly, Applicants respectfully request that this rejection be withdrawn.

The rejection of claims 1, 2, 18, 58-60, 76 and 79-99 under 35 U.S.C. § 102(b) as allegedly anticipated by Krainc et al., *J. Biol. Chem.* 273:26218-26224 (1998), is respectfully traversed. Although Applicants maintain that the claims are novel over Krainc et al., in an effort to further prosecution, claims 1-20 and 58-79 are cancelled herein rendering the rejection of these claims moot. Applicants herein will address the above rejections as they apply to pending claims 80-99. Applicants respectfully maintain that the claimed methods are novel over Krainc et al. based on the arguments of record and in view of the following.

Anticipation requires that "each element of the claim at issue is found, either expressly described or under the principles of inherency, in a single prior art reference or that the claimed invention was previously known or embodied in a single prior art device or practice." *Kalman v. Kimberly-Clark Corp.*, 713 F.2d 760, 771 (Fed. Cir. 1983). See *MEHL/Biophile Int'l Corp. v Milgraum*, 192 F.3d 1362, 1365 (Fed. Cir. 1999) (to anticipate, a single reference must teach every limitation of the claimed invention; any limitation not explicitly taught must be inherently taught and would be so understood by a person experienced in the field); *In re Baxter Travenol Labs.*, 952 F.2d 388, 390 (Fed. Cir. 1991) (the dispositive question is "whether one skilled in the art would reasonably understand or infer" that a reference teaches or discloses all of the elements of the claimed invention); *Continental Can Co. v. Monsanto Co.*, 948 F.2d 1264, 1268-69 (Fed. Cir. 1991) (to anticipate, every element of the claims must appear in a single prior art reference, or if not expressly shown, then demonstrated to be known to persons experienced in the field of technology); *In re Samour*, 571 F.2d 559, 562 (CCPA 1978) (the key question is whether a single prior art reference "publicly discloses every material element of the claimed subject matter").

In the Office Action on page 21, Krainc et al. is described as disclosing that the plasmid pG/DNA, containing the N-terminal DNA binding domain of MEF2C, was stably transected into

P19 cells, referring to Figure 5 and page 26222, column 2, paragraph 2. The referenced paragraph from Krainc et al. reads as follows:

> To gain more direct evidence that endogenous MEF2C is involved in *NR1* gene expression *in vivo*, we monitored NR1 mRNA levels during neuronal differentiation in the presence or absence of a dominant-negative MEF2C protein. We stably transfected the plasmid pG/DN, which contains the cDNA sequence of the NH_2-terminal DNA binding domain of MEF2C, into P19 cells. These cells differentiate into a neuronal phenotype after treatment with 13-*cis*-retinoic acid, and then express MEF2C (55) as well as glutamate receptor mRNAs (38). By reverse transcriptase-PCR, we observed induction of NR1 mRNA expression in P19 cells after differentiation with 13-*cis*-retinoic acid for 7 days (Fig. 5A, lane 4). This induction was totally abolished, however, in p19 cells stably expressing the dominant-negative MEF2C (Fig. 5A, lane 5). [emphasis added]

As described in the passage above and on page 26219, column 1, under "Stable Transfection," the pGK/DN construct contains a dominant-negative MEF2C cDNA. In contrast, the claimed methods include the step of introducing into the progenitor cells a nucleic acid molecule encoding a constitutively active MEF2 polypeptide or an active fragment thereof, thereby differentiating the progenitor cells to produce a cell population containing neuronal cells protected from apoptotic cell death. Krainc et al. provides no teaching of introducing into progenitor cells a nucleic acid molecule encoding a constitutively active MEF2 polypeptide or an active fragment thereof to produce a cell population containing neuronal cells protected from apoptotic cell death. Furthermore, the dominant-negative MEF2C cDNA of Krainc et al., when introduced into p19 cells totally abolished the cells from differentiating into a neuronal phenotype following treatment with retinoic acid, as described in the passage above. Applicants respectfully submit that Krainc et al. cannot anticipate the claimed methods as Krainc et al. do not teach, either expressly or inherently, each element of the claimed methods. Accordingly, Applicants respectfully request that this rejection be withdrawn.

The rejection of claims 1, 2, 18, 58-60, 76 and 79-99 under 35 U.S.C. § 102(b) as allegedly anticipated by Skerjanc et al., *FEBS Lett.* 472:53-63 (2000) (hereinafter Skerjanc et al., (2000)), as evidenced by Skerjanc et al., *J. Biol. Chem.* 273:34904-34910 (1998) (hereinafter Skerjanc et al., (1998)), is respectfully traversed. Although Applicants maintain that the claims are novel over Skerjanc et al. (2000) as evidenced by Skerjanc et al. (1998), in an effort to further prosecution, claims 1-20 and 58-79 are cancelled herein rendering the rejection of these

claims moot. Applicants herein will address the above rejections as they apply to pending claims 80-99. Applicants respectfully maintain that the claimed methods are novel over Skerjanc et al., (2000) alone or in combination with Skerjanc et al. (1998) based on the arguments of record and in view of the following.

The Office Action on page 23 asserts that Skerjanc et al. (2000) describe that mouse P19 cell lines overexpressing MEF2C differentiated into neural cells in the presence of DMSO (abstract; page 54, column 2, paragraph 2; and Figure 2). The Office Action goes on to assert that at page 53, column 2, paragraph 3, Skerjanc et al. (2000) describes that P19 cells overexpressing MEF2C, termed p19[MEF2C] cells, were described previously by Skerjanc et al. (1998). Applicants respectfully direct the Office to page 34905, left column, under the heading "Plasmid Constructs" of Skerjanc et al. (1998), wherein the plasmid construct encoding the MEF2C polypeptide is described:

> The DNA construct PGK-MEF2C contains the phosphoglycerate kinase (*pgk-1*) promoter (51) driving the coding region of human MEF2C (20). This isoform of MEF2C binds DNA and activates transcription.

Reference (20) is Leifer et al., *Proc. Natl. Acad. Sci. USA* 90:1546-1550 (1993), provided herein as Exhibit B. Leifer et al. describe isolation of human MEF2C, the sequence of which was described to have been deposited in the GenBank database (accession no. L08895) (see page 1546, left column, second paragraph and footnote "**"). Applicants direct the Office to the subject application, which teaches on page 10, lines 20-24 that nucleotide sequence SEQ ID NO: 5 of human MEF2C corresponds to the same GenBank accession no. L08895. This is the native, i.e. wild type MEF2C polypeptide. The subject application defines a constitutively active MEF2 polypeptide on page 40, line 17 to page 41, line 31:

> While native MEF2 polypeptides are activated through phosphorylation, for example, by p38 MAP kinase, constitutively active forms of MEF2 do not require such phosphorylation for activation. Any of the methods of the invention can be practiced using a constitutively active MEF2 polypeptide to induce the p38/MEF2 pathway. [emphasis added]
>
> As used herein in reference to a MEF2 polypeptide, the term "constitutively active" means a MEF2 polypeptide that has transactivation activity which is less dependent upon phosphorylation than the corresponding wild type MEF2 polypeptide. A constitutively active MEF2 polypeptide can have transactivation

> activity that is independent of phosphorylation. As disclosed herein, a MEF2 polypeptide can be cleaved by a caspase to produce a dominant negative form of a MEF2 polypeptide having pro-apoptotic activity. In one embodiment, a constitutively active form of a MEF2 polypeptide is resistant to caspase cleavage. [emphasis added]
>
> A constitutively active MEF2 polypeptide can include, for example, a heterologous transactivation domain in addition to, or in place of, the native MEF2 transactivation domain. A constitutively active MEF2 polypeptide can be, for example, a MEF2A, MEF2B, MEF2C or MEF2 D polypeptide containing a GAL4 or VP16 transactivation domain in addition to, or in place of, the native MEF2 transactivation domain. In specific embodiments, a constitutively active MEF2 polypeptide is a chimera in which the native MEF2 activation domain is replaced with a heterologous activation domain, for example, a constitutively active MEF2A/VP16, MEF2A/GAL4, MEF2B/VP16, MEF2B/GAL4, MEF2C/VP16, MEF2C/GAL4, MEF2D/VP16 or MEF2D/GAL4 fusion protein. [emphasis added]
>
> A constitutively active MEF2 polypeptide also can be a MEF2 polypeptide in which the native activation domain is modified such that transactivation does not depend on phosphorylation. A constitutively active MEF2 polypeptide can have, for example, one or modified phosphorylation sites within the transactivation domain, for example, one or more serine/threonine to aspartic acid/glutamic acid amino acid substitutions within the transactivation domain. See, for example, Watson et al., J. Neurosci. 18:751-762 (1998), which demonstrates that mutation of the Jun kinase phosphorylation site in c-Jun to aspartic acid produces a constitutively active c-Jun polypeptide that is independent of Jun kinase. [emphasis added]

Applicants submit that the MEF2C overexpressed in mouse P19 cells by Skerjanc et al. (2000) was not a constitutively active MEF2 polypeptide or an active fragment thereof as recited in the pending claims, but was the native, i.e. wild type, MEF2C polypeptide. Applicants respectfully submit that Skerjanc et al. (2000) as evidenced by Skerjanc et al. (1998) cannot anticipate the claimed methods because neither reference teach, either expressly or inherently, each element of the claimed methods. Accordingly, Applicants respectfully request that this rejection be withdrawn.

CONCLUSION

In light of the amendments and remarks herein, Applicants submit that the claims are now in condition for allowance and respectfully request a notice to this effect. The Examiner is invited to call the undersigned agent if there are any questions.

To the extent necessary, a petition for an extension of time under 37 C.F.R. 1.136 is hereby made. Please charge any shortage in fees due in connection with the filing of this paper, including extension of time fees, to Deposit Account 502624 and please credit any excess fees to such deposit account.

Respectfully submitted,

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Exhibit A

blood 1998 92: 4013-4022

Highly Efficient Transduction of the Green Fluorescent Protein Gene in Human Umbilical Cord Blood Stem Cells Capable of Cobblestone Formation in Long-Term Cultures and Multilineage Engraftment of Immunodeficient Mice

Paula B. van Hennik, Monique M.A. Verstegen, Marti F.A. Bierhuizen, Ana Limón, Albertus W. Wognum, José A. Cancelas, Jordi Barquinero, Rob E. Ploemacher and Gerard Wagemaker

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RAPID COMMUNICATION

Highly Efficient Transduction of the Green Fluorescent Protein Gene in Human Umbilical Cord Blood Stem Cells Capable of Cobblestone Formation in Long-Term Cultures and Multilineage Engraftment of Immunodeficient Mice

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Purified CD34$^+$ and CD34$^+$CD38$^-$ human umbilical cord blood (UCB) cells were transduced with the recombinant variant of Moloney murine leukemia virus (MoMLV) MFG-EGFP or with SF-EGFP, in which EGFP expression is driven by a hybrid promoter of the spleen focus-forming virus (SFFV) and the murine embryonic stem cell virus (MESV). Infectious MFG-EGFP virus was produced by an amphotropic virus producer cell line (GP+*env*Am12). SF-EGFP was produced in the PG13 cell line pseudotyped for the gibbon ape leukemia virus (GaLV) envelope proteins. Using a 2-day growth factor prestimulation, followed by a 2-day, fibronectin fragment CH-296–supported transduction, CD34$^+$ and CD34$^+$CD38$^-$ UCB subsets were efficiently transduced using either vector. The use of the SF-EGFP/PG13 retroviral packaging cell combination consistently resulted in twofold higher levels of EGFP-expressing cells than the MFG-EGFP/Am12 combination. Transplantation of 10^5 input equivalent transduced CD34$^+$ or 5×10^3 input equivalent CD34$^+$CD38$^-$ UCB cells in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice resulted in median engraftment percentages of 8% and 5%, respectively, which showed that the in vivo repopulating ability of the cells had been retained. In addition, mice engrafted after transplantation of transduced CD34$^+$ cells using the MFG-EGFP/Am12 or the SF-EGFP/PG13 combination expressed EGFP with median values of 2% and 23% of human CD45$^+$ cells, respectively, which showed that the NOD/SCID repopulating cells were successfully transduced. EGFP$^+$ cells were found in all human hematopoietic lineages produced in NOD/SCID mice including human progenitors with in vitro clonogenic ability. EGFP-expressing cells were also detected in the human cobblestone area–forming cell (CAFC) assay at 2 to 6 weeks of culture on the murine stromal cell line FBMD-1. During the transduction procedure the absolute numbers of CAFC week 6 increased 5- to 10-fold. The transduction efficiency of this progenitor cell subset was similar to the fraction of EGFP$^+$ human cells in the bone marrow of the NOD/SCID mice transplanted with MFG-EGFP/Am12 or SF-EGFP/PG13 transduced CD34$^+$ cells, ie, 6% and 27%, respectively. The study thus shows that purified CD34$^+$ and highly purified CD34$^+$CD38$^-$ UCB cells can be transduced efficiently with preservation of repopulating ability. The SF-EGFP/PG13 vector/packaging cell combination was much more effective in transducing repopulating cells than the MFG-EGFP/Am12 combination.

EFFICIENT PROCEDURES for gene transfer into human immature hematopoietic cells with repopulating capacities after transplantation may in principle open new avenues for the treatment of a variety of hereditary and acquired diseases. Retroviral-mediated gene transfer to such cells, which is attractive by its simplicity and efficiency, has, however met with considerable difficulty, which is only partly understood.[1,2] The availability of a rapid selectable marker, such as the green fluorescent protein (GFP), is thought to be of pivotal importance to study major variables influencing the efficiency of gene transfer, as well as to track the progeny of transduced cells after transplantation. In the present study we evaluated the use of the enhanced (E) recombinant variant of GFP to label immature human umbilical cord blood cells, using outgrowth in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice[1,3,4] as well as cobblestone area–forming cells (CAFC)[5,6] as assays for immature cells with considerable hematopoietic reconstitution capacity.

The CAFC assay and the long-term culture-initiating cell (LTC-IC) assay allow for frequency analysis of cells capable of long-term repopulation in vitro.[5,7] Murine studies have shown that the CAFC scored at week 2 are related to colony-forming unit-spleen (CFU-S) day 12, while CAFC week 5 strongly correlate with long-term repopulating cells in vivo.[6,8] In human hemopoiesis the rare population with the primitive phenotype of CD34$^+$CD38$^-$ is highly enriched for CAFC week 6. The primitive nature of CAFC week 6 is further illustrated by enrichment after incubation with 5-fluorouracil (5-FU), a drug cytotoxic for proliferating cells. The CAFC week 2, however, are absent in the CD34$^+$CD38$^-$ population and more than 1 log reduced after 5-FU treatment. Based on these results, the CAFC week 6 have been proposed to be representative for cells with long-term repopulating ability in vivo in the human situation.[9] On this basis, this assay is considered suitable to assess the effect of manipulation of human hematopoietic progenitor cell populations, such as by gene-transfer protocols.[10,11]

The efficiency of gene transfer to stem cells is limited by the inability of most retroviral vectors to integrate DNA into the

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cellular genome of quiescent cells.[12-15] Stimulation of stem-cell cycling with hematopoietic growth factors (HGF) such as interleukin-3 (IL-3), IL-6, stem cell factor (SCF), or Flt3-L[16] before and during virus exposure would seem to be essential to promote transduction,[17,18] but may result in loss of repopulating ability of transduced cells as a result of differentiation.[16,19] In addition, colocalization of target cells and virus on dishes coated with the recombinant fibronectin-fragment CH-296 has been shown to further increase gene transfer efficiency.[20,21]

For transduction of human hematopoietic cells, murine retroviruses based on the Moloney murine leukemia virus (MoMLV) are most commonly used. However, expression of functional receptors for the MoMLV envelope protein is presumably low, and pseudotyping the vector with the GaLV envelope protein resulted in higher transduction efficiencies in hematopoietic progenitor cells,[22-24] which has been attributed to a higher expression of functional pseudotyped GaLV receptor (Pit-1) by the immature hematopoietic cells[22,24] than the amphotropic retroviral receptor (Pit-2).[24-28] A study in which $CD34^+$ cells were transduced by the GaLV-pseudotyped retroviral vector showed that $CD34^+$ cells were efficiently transduced (21% to 33% transduction) as determined by culture in a colony-forming cell assay.[2] It is not known to what extent the relative transduction inefficiency of the MoMLV type viruses is caused by a low Pit-2 expression on immature stem cells or by inefficient activation and provirus integration in quiescent cells. Transplantation of $CD34^+$ or $CD34^+CD38^-$ transduced cells in immunodeficient beige/nude/xid (bnx) mice showed that 8 of 10 mice transplanted with $CD34^+$ transduced cells contained the retrovirally transduced bacterial neomycin phosphotransferase resistance (neo), gene whereas only 2 of 14 mice that had received $CD34^+CD38^-$ cells contained low levels of transduced cells.[2] The ability to engraft the bone marrow (BM) of NOD/SCID mice and provide multilineage outgrowth, which resides exclusively in the $CD34^+CD38^-$ population,[3] has been described as unsuccessful, in contrast to the LTC-IC or CAFC week 6, which were transduced with efficiencies ranging between 10% and 70%.[1] These differences led to the suggestion that NOD/SCID repopulating cells are distinct from the LTC-IC or CAFC week 6.[1] However, recent data obtained with vectors that contained the neo-gene show that transplantation of retrovirally transduced $CD34^+$ UCB cells in NOD/SCID mice result in transduced human hematopoiesis in the NOD/SCID BM with transduction levels similar to those obtained for LTC-IC.[29]

Use of the GFP gene from the jellyfish *Aequorea victoria* as a retrovirally transduced marker allows rapid identification of transduced cells by fluorescence microscopy, flow cytometry, or culture in real time without additional staining steps in contrast to other genetic markers such as the neo-gene[30-32] and the bacterial β-galactosidase gene (LacZ).[33-36] As wild-type GFP produces a weak (but stable) green fluorescence signal, several GFP variants, such as EGFP, have been created which are better suited for detection of expression by fluorescence microscopy and flow cytometry.[37,38] Studies with murine cells have shown that cells with the ability of in vivo reconstitution can be transduced with EGFP.[39] Our ongoing studies show that high expression levels of EGFP could be detected in mouse BM, peripheral blood, spleen, and thymus for a current observation period of 6 months after transplantation and were retained in secondary recipient mice, indicating that long-term repopulating stem cells can be successfully transduced. Human cell lines and purified $CD34^+$ cells were also transduced using EGFP-containing vectors.[28] Therefore, retroviral vectors containing EGFP genes can be used to transduce a variety of cells, which can then be easily detected in vitro as well as in vivo.

To initiate an analysis directed at optimal vectors and transduction procedures, the MFG-EGFP retroviral vector produced by an amphotropic packaging cell line and the SF-EGFP vector pseudotyped for the GaLV envelope protein were used to transduce immature cell subsets in human umbilical cord blood (UCB). The potential of these vector/packaging cell combinations for transduction of purified $CD34^+$ and $CD34^+CD38^-$ UCB subsets was compared by assessing the ability of transduced cells to produce $EGFP^+$ cobblestone areas in the CAFC assay and to contribute to multilineage human hematopoiesis in NOD/SCID mice.

MATERIALS AND METHODS

Human UCB cells. UCB samples were obtained from placentas of full-term normal pregnancies after informed consent in conformity with legal regulations in The Netherlands. Mononucleated cells were isolated by Ficoll density gradient centrifugation (1.077 g/cm^2; Nycomed Pharma AS, Oslo, Norway), and were cryopreserved in 10% dimethylsulphoxide, 20% heat-inactivated fetal calf serum (FCS), and 70% Hanks' Balanced Salt Solution (HBSS; GIBCO, Breda, The Netherlands) at −196°C as described[40] before use. After thawing by stepwise dilution in HBSS containing 2% FCS, the cells were washed with HBSS containing 1% FCS and used for gene transduction experiments.

Viral vectors and packaging cell lines. The amphotropic retroviral producer cell line, MFG-EGFP, was obtained by a 20-hour incubation of GP+*env*Am12 under standard culture conditions with supernatants containing ecotropic retrovirus from the GP+E-86/MFG-EGFP cell line and hexadimethrine bromide at 4 μg/mL (Sigma, St Louis, MO) as described.[38] The pseudotyped retroviral producer cell line PG13/EGFP7 was developed by transducing the PG13 packaging cell line (kindly provided by D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA) with 0.45 μm filtered supernatant from PA317/EGFP cell cultures.[28] EGFP expression was analyzed by flow cytometry and bright single cells were sorted on 96-well plates by using an EPICS Elite ESP flow cytometer coupled to an autoclone device (both from Coulter, Miami, FL). Single clones were cultured as previously described.[28] The sorted clones were additionally selected for high virus titer. The viral titer of both the amphotropic and the pseudotyped producer cell line was in the order of 10^6 infectious particles per mL as determined by supernatant titration on cultured murine NIH 3T3 cells and human HeLa cells, respectively. Absence of replication-competent virus was verified by failure to transfer GFP expression from a transduced cell population to a secondary population. Additionally, for the SF-EGFP/PG13 vector/packaging cell combination pseudotransduction was tested on HeLa cells and found absent.

Subset purification. Purification of $CD34^+$ cells was performed by positive selection using Variomacs Immunomagnetic Separation System as described[41] (CLB, Amsterdam, The Netherlands). The percentage of $CD34^+$ cells in the unseparated population (low-density UCB) and in the purified $CD34^+$ and $CD34^-$ fractions was determined by fluorescence-activated cell sorting (FACS) analysis. For isolation of $CD34^+CD38^-$ subsets, purified $CD34^+$ cells were stained with fluorescein isothiocyanate (FITC) and R-phycoerythrin (PE) conjugated antibodies against human CD34 and CD38 (CD34-FITC, CD38-PE; Becton Dickinson, San Jose, CA) for 30 minutes on ice in HBSS, supplemented with 2% (wt/vol) bovine serum albumin (BSA; Sigma), 0.05% (wt/vol) sodium azide (Merck, Darmstadt, Germany) and 2%

(vol/vol) normal human serum (NHS). After incubation, the cells were washed twice, resuspended in HBSS and $CD34^+CD38^-$ cells, and the window set at 5% of the $CD34^+$ population with the lowest CD38 expression levels (Fig 1) were sorted using a FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA).

Retroviral transduction of UCB subsets. Supernatants containing recombinant retrovirus were generated by culturing approximately 80% confluent producer cells for 12 hours in culture medium consisting of a serum-free enriched version of Dulbecco's modified Eagle's medium (DMEM; GIBCO, Gaithersburg, MD).[3,39,42] Media for all cultures routinely included 100 U/mL of penicillin and 100 μg/mL of streptomycin. The cultures were maintained at 37°C with 10% CO_2 (measured every 15 minutes with read-outs between 9.5% and 10%) in a humidified atmosphere. The culture supernatant was subsequently procured and passed through a 0.45-μm filter. To enhance the transfection efficiency, Falcon 1008 (35-mm) bacteriological culture dishes (Becton Dickinson, Plymouth, UK) were coated with the recombinant fibronectin fragment CH-296 (Takara Shuzo, Otsu, Japan) at a concentration of 10 μg/cm^2 as described previously.[21] UCB subsets ($CD34^+$ or $CD34^+CD38^-$) were prestimulated for 2 days in either medium consisting of enriched Dulbecco's medium (GIBCO, Gaithersburg, MD), or CellGroSCGM (Boehringer Ingelheim, Heidelberg, Germany). Different combinations of human recombinant HGF were added to the culture medium; IL-3 (20 ng/mL; Gist-brocades NV, Delft, The Netherlands), IL-6 (100 ng/mL; Ares-Serono SA, Genève, Switzerland), thrombopoietin (TPO; 10 ng/mL, kindly provided by Genentech, South San Francisco, CA), SCF (100 ng/mL), and Flt3-L (50 ng/mL; the latter two kindly provided by Amgen, Thousand Oaks, CA). The HGF combination of Flt-3L, TPO, IL-6, and SCF was used during the transduction procedure; in some initial experiments, as indicated in the legend of the figures and tables, the IL-3, IL-6, SCF combination was used. Before adding purified cord blood subsets to the fibronectin-coated dishes, the CH-296 fibronectin fragment was preincubated with supernatant containing the amphotropic MFG-EGFP or the pseudotyped SP-EGFP vector for 1 hour at 37°C.[20,21] Subsequently, nucleated cells were resuspended in the vector-containing supernatant supplemented with hematopoietic growth factors and added to the dishes. Over a period of 2 days, culture supernatant was once replaced completely by resuspending nonadherent cells into fresh retrovirus supernatant and HGF. Finally, the cells were obtained and used for FACS analysis, human granulocyte-macrophage CFU (GM-CFU) and erythroid burst-forming units (BFU-E) assays, CAFC assay, and transplantation into NOD/SCID mice.

Flow cytometry. Cell samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson) as previously described.[38,39] Immunophenotyping of EGFP-transduced cells was performed by staining with peridinin chlorophyll protein (PercP)-labeled anti-CD45 and cyanin-5–conjugated anti-CD34 (Cy5; Amersham, Buckinghamshire, UK) or PE-conjugated monoclonal antibodies against CD38, CD2, CD4, CD8, CD19, CD20, CD56, CD33 (Becton Dickinson). Mice were considered engrafted if the percentage $CD45^+$ cells exceeded 1%.

Transplantation of transduced UCB subsets in immunodeficient mice. Specific pathogen-free (SPF) NOD/LtSz-scid/scid (NOD/SCID) mice, 6 to 9 weeks of age, were bred and housed under SPF conditions in a laminar air flow unit and supplied with sterile food and acidified drinking water containing 100 mg/L ciprofloxacine (Bayer AG, Leverkusen, Germany) ad libitum. Housing, care, and all animal experimentation were done in conformity with legal regulations in The Netherlands, which include approval by a local ethical committee. All mice received total body irradiation (TBI) at 3.5 Gy, delivered by a ^{137}Cs source adapted for the irradiation of mice (Gammacell, Atomic Energy of Canada, Ottawa), 2 to 4 hours before transplantation. The transplants were suspended in 200 μL HBSS containing 0.1% BSA and injected intravenously (IV) into a lateral tail vein. Transplanted cell numbers were 10^5 $CD34^+$ cells and 5×10^3 $CD34^+CD38^-$ cells. Thirty-five days after transplantation the mice were killed by CO_2 inhalation followed by cervical dislocation, both femurs isolated, and BM cell suspensions prepared by flushing. After counting, the cells were cultured in colony assays and analyzed by flow cytometry to determine the percentage of human $EGFP^+$ cells in the mouse BM.

In vitro colony assay. Purified UCB cells, EGFP-transduced cells, and chimeric mouse BM samples were assayed for the presence of human GM-CFU and BFU-E by in vitro colony formation in viscous methylcellulose culture medium as previously described.[3,42-44] The number of colonies was determined after 14 days of culture in a humidified atmosphere of 10% CO_2 at 37°C. $EGFP^+$ colonies were scored under excitation by UV light.

Stromal feeders and CAFC assay. The contact inhibited FBMD-1 murine stromal cell line was used as described before.[5] After 7 to 10 days of culture at 33°C and 10% CO_2, the stromal layers had reached confluence and were overlaid with nontransduced or transduced $CD34^+$ or $CD34^+CD38^-$ UCB cells within the subsequent week. Confluent

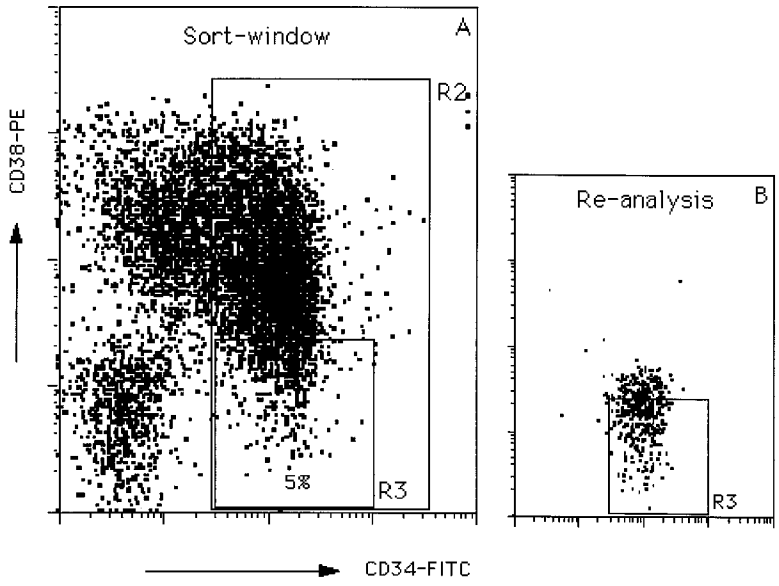

Fig 1. Flow cytometric profile used to define and sort the $CD34^+CD38^-$ cell population (A). The window R3 was used to define $CD34^+CD38^-$ cells for sorting and contains 5% of the $CD34^+$ population (as defined by window R2) with the lowest CD38 antigen expression. Re-analysis of the sorted cells is shown in (B).

stromal layers of FBMD-1 cells in flat-bottom 96-well plates were overlaid with UCB cells in a limiting dilution setup. Input values of the $CD34^+CD38^-$ population and the $CD34^+$ were 25 nucleated cells and 500 nucleated cells per well in the first dilution, respectively. Twelve twofold serial dilutions were used for each sample with 15 replicate wells per dilution. The cells were cultured at 33°C and 10% CO_2 for 6 weeks with weekly half-medium changes. The percentage of wells with at least one phase-dark hematopoietic clone of at least five cells (ie, a cobblestone area) beneath the stromal layer was determined weekly with an inverted microscope. Green fluorescent cobblestone areas were screened in the same way but with a UV-light excitation source. Frequencies of total and green-fluorescent CAFC were calculated by using Poisson statistics as described previously.[6] During the period of culture, no transfer of the EGFP gene to the stromal underlayer has been observed.

Statistical analysis. Data are expressed as median (range). Statistical comparisons were performed according to Mann Whitney U-test. P values $<.05$, two-tailed, were considered significant.

RESULTS

Transduction efficiencies in purified cells with MFG-EGFP and SF-EGFP vectors. Purified $CD34^+$ and $CD34^+CD38^-$ UCB cells (Fig 1) were prestimulated for 2 days and subsequently transduced with either MFG-EGFP/Am12 or SF-EGFP/PG13 vector/packaging cell combination, during 2 days of exposure to virus-containing supernatants in fibronectin fragment-coated bacterial dishes. Transduction efficiencies obtained by infection using the amphotropic MFG-EGFP producer cell line were compared to those obtained with the pseudotyped SF-EGFP cell line. The percentage $EGFP^+$ cells was assessed by flow cytometry (Fig 2). The percentage of $EGFP^+$ cells of the purified $CD34^+$ population transduced with the SF-EGFP/PG13 vector/packaging cell combination (median, 75% $EGFP^+$) was more than twofold higher compared with MFG-EGFP/Am12–transduced $CD34^+$ cells (median, 30%) (Table 1). Sorted $CD34^+CD38^-$ cells were also transduced at a higher frequency using the SF-EGFP/PG13 combination (62%) than after transduction with the MFG-EGFP/Am12 combination (19%). On average, transduction frequencies were lower in the purified $CD34^+CD38^-$ cells than in the $CD34^+$ cell fraction, but only for the MFG-EGFP/Am12–transduced cells the difference was statistically significant. The level of transduction of the $CD34^+CD38^-$ subset within the purified $CD34^+$ population obtained with the SF-EGFP/PG13 vector/packaging cell combination was more than 2.5-fold higher than with the MFG-EGFP/Am12 combination. The differences in transduction efficiency between the two vector/packaging cell combinations in these cell populations were significant ($P < .025$).

Transduction efficiency of CAFC subsets. The ability of transduced cells to form cobblestone areas was evaluated in long-term culture supported by FBMD-1 stromal cells. $EGFP^+$ cobblestone areas were identified by fluorescence microscopy (Fig 3). The absolute numbers of CAFC at different culture periods increased as a result of the transduction procedure without significant differences between the target cells or vector used (Table 2). The absolute number of CAFC week 2 in the MFG-EGFP/Am12–transduced $CD34^+$ UCB cells increased 5-fold, for the SF-EGFP/PG13–transduced $CD34^+$ UCB cells the increase was 7-fold. The CAFC week 6 expanded 10-fold and 5-fold, respectively. For the $CD34^+CD38^-$ UCB cells, similar results were obtained, 6-fold and 10-fold of CAFC week 6 after MFG-EGFP/Am12 and SF-EGFP/PG13 transduction, respectively. Consistent with the immaturity of the $CD34^+CD38^-$ cell population, CAFC week 2 could not be detected in the $CD34^+CD38^-$ cell fraction before transduction. These data show that the transduction protocol that has been used causes a modest expansion of both CAFC week 2 and week 6.

The transduction efficiency of the CAFC week 2 in MFG-EGFP/Am12 transduced $CD34^+$ cells ranged between 23% and 30% with a median value of 26%, and in SF-EGFP/PG13–transduced $CD34^+$ cells the median value was 60% (46% to 74%) (Table 2). The transduction efficiency of the CAFC week 6 in MFG-EGFP/Am12–transduced $CD34^+$ cells ranged between 0% and 11% with a median of 6% $EFGP^+$ cobblestone areas. CAFC week 6 in SF-EGFP/PG13–transduced $CD34^+$ cells showed transduction as high as 27%. CAFC week 6 in SF-EGFP/PG13–transduced $CD34^+CD38^-$ cells showed a similar level of 25% transduction efficiency. Notably, highly purified $CD34^+CD38^-$ cells transduced with the amphotropic cell line did not produce $EGFP^+$ cobblestone areas week 6. These experiments clearly showed the superiority of SF-EGFP/PG13 over MFG-EGFP/Am12 in transducing late appearing CAFC, in concordance with the results obtained in phenotypically identified immature $CD34^+$ subsets.

Repopulation of transduced subsets in NOD/SCID mice. In parallel with analysis of cobblestone formation, the ability of transduced cells to reconstitute hematopoiesis in vivo was examined by transplantation of the equivalent of 10^5 noncultured $CD34^+$ cells into sublethally irradiated NOD/SCID mice. After 35 days the level of chimerism and the percentage of $EGFP^+$ cells in mouse BM were determined by flow cytometry (Table 3). Similar levels of engraftment were found in mice transplanted with noncultured or cultured $CD34^+$ cells. After

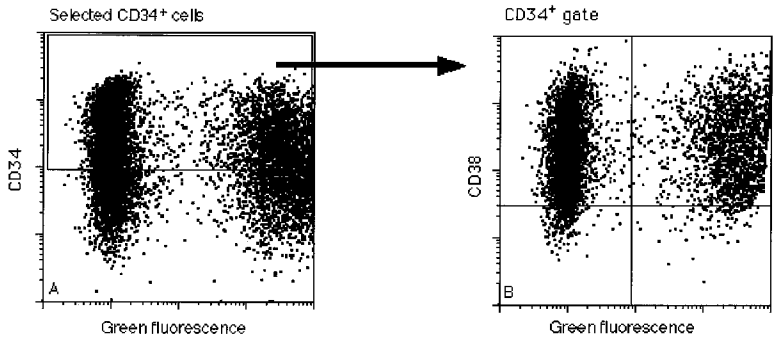

Fig 2. Flow cytometric analysis of a representative transfection of purified $CD34^+$ cells with the amphotropic MFG-EGFP retroviral vector after 2 days of prestimulation and 2 days of supernatant infection in the presence of IL-3, IL-6, and SCF. This particular transduction resulted in efficiencies of 30% within the $CD34^+$ population (A). In (B) $CD34^+$ cells were gated and the CD38 distribution of the EGFP-transduced cells was studied. Also, $CD34^+CD38^-$ cells expressed the EGFP gene with efficiencies similar to the total $CD34^+$ population (30% $EGFP^+$).

Table 1. EGFP Expression of UCB Subsets

Vector/Packaging Cell Line	Purified $CD34^+$ Cells	*P* Value*	$CD34^+CD38^-$ Population Within Purified $CD34^+$	*P* Value†	Purified $CD34^+CD38^-$	*P* Value‡
MFG-EGFP/Am12	30 (8-51) (n = 13)	>.05	25 (15-55) (n = 9)	>.05	19 (8-21) (n = 4)	.02
SF-EGFP/PG13	75 (53-84) (n = 7)	>.05	66 (58-81) (n = 5)	>.05	62 (21-71) (n = 4)	.12
P value§	.0001		.003		.02	

Results are expressed as percentages of $EGFP^+$ cells and depicted as median (range). For statistical analysis the Mann-Whitney U-test has been used.

*Comparison of the median of purified $CD34^+$ cells and $CD34^+CD38^-$ subset within the purified $CD34^+$ population.

†Comparison of the median of $CD34^+CD38^-$ subset within the purified $CD34^+$ population and purified $CD34^+CD38^-$ cells.

‡Comparison of the median of purified $CD34^+$ cells and purified $CD34^+CD38^-$ cells.

§Comparison of MFG-EGFP– and SF-EGFP–transduced cells.

transplantation of noncultured $CD34^+$ cells human cells were detected in all mice (n = 11) (median, 54% [range, 6% to 64%] $CD45^+$ cells). $EGFP^+$ cells were found in 6 of 10 repopulated chimeric mice transplanted with MFG-EGFP/Am12–transduced $CD34^+$ cells with a median percentage of $EGFP^+$ cells of 2% (Table 3). $CD34^+$ cells transduced using the SF-EGFP/PG13 vector produced higher levels of $EGFP^+$ cells (median, 23%) in the human population in all four mice transplanted. These data showed that the repopulating cells in the $CD34^+$ population can be transduced effectively and produce $EGFP^+$ progeny in transplanted NOD/SCID mice. In addition, SF-EGFP/PG13 was much more efficient in transducing the repopulating cells than MFG-EGFP/Am12.

Transplantation of noncultured $CD34^+CD38^-$ cells and transduced $CD34^+CD38^-$ resulted in chimerism levels of median 10% (range, 6% to 29%) for the noncultured cells and 5% (range, 1% to 24%) and 6% (range, 4% to 9%) for the MFG-EGFP/Am12– or SF-EGFP/PG13–transduced cells, re-

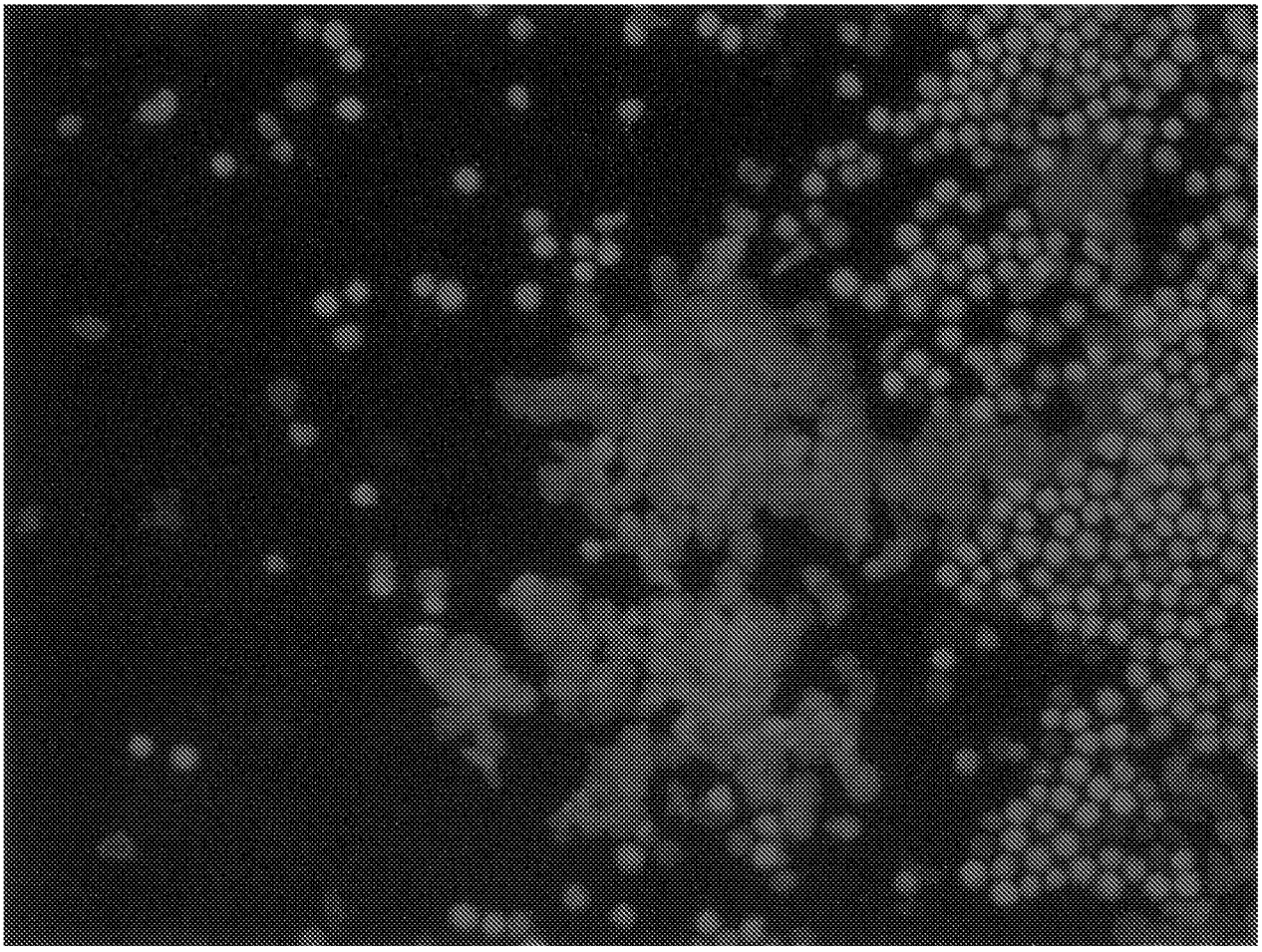

Fig 3. Fluorescence microscopic image of a representative $EGFP^+$ cobblestone area. The bright green cells are the mature cells on top of the stromal layer and the dim green cells represents the $EGFP^+$ cobblestone area.

Table 2. Absolute Numbers of CAFC Week 2 and Week 6 and Percentages of Green Fluorescent Cobblestone Areas After Transduction of 10^6 Selected UCB CD34$^+$ Cells or 35×10^3 CD34$^+$CD38$^-$ Cells With the Vectors MFG-EGFP or SF-EGFP

	CAFC wk 2				CAFC wk 6			
	CD34$^+$	%	CD34$^+$CD38$^-$	%	CD34$^+$	%	CD34$^+$CD38$^-$	%
Before transduction	42×10^3	—	ND	—	4×10^3	—	0.3×10^3	—
MFG-EGFP/Am12	218×10^3*	26	2×10^3†	15	41×10^3*	6	2×10^3†	ND
SF-EGFP/PG13	315×10^3*	60	2×10^3†	24	22×10^3*	27	3×10^3†	25

Abbreviations: %, percentage of green fluorescent cobblestone areas expressed as median; ND, not detectable.
*n = 2.
†n = 1.

spectively. In contrast to the results with purified CD34$^+$ cells, CD34$^+$CD38$^-$ cells transduced with MFG-EGFP/Am12 were not able to repopulate mouse BM with EGFP-expressing cells, although all four mice engrafted with human cells (Table 2); this parallels the absence of EGFP expressing CAFC week 6 in CD34$^+$CD38$^-$ cells transduced with MFG-EGFP/Am12. Only one of three mice engrafted with SF-EGFP/PG13–transduced CD34$^+$CD38$^-$ cells. EGFP$^+$ could only be detected in 3% of the CD45$^+$ cells produced. This is in contrast to the results with the CD34$^+$ cells in that apparently most repopulating cells in the highly purified CD34$^+$CD38$^-$ subset were not transduced efficiently or the transduced cells displayed a significant reduction in their engraftment potential compared with the cells that were not transduced during the procedure. Nevertheless, SF-EGFP/PG13 in these experiments was also apparently more efficient than MFG-EGFP/Am12.

Multilineage outgrowth of EGFP-transduced CD34$^+$ cells. The composition of the EGFP$^+$ human cell population in two mice was assessed by flow cytometry using a panel of lineage-specific markers (Fig 4). EGFP$^+$ cells of the myeloid lineage (CD33, range, 31% to 39%; CD11b, range, 20% to 25%; CD4, range, 30% to 45%), T-lymphoid (CD2, range, 20% to 22%), B-lymphoid (CD20, range, 16% to 23%), and natural killer (NK) cells (CD56, 1%) were found in mice transplanted with EGFP-transduced CD34$^+$ cells. Also, immature EGFP$^+$CD34$^+$ cells were present in the mouse BM (range, 1.1% to 6.8%) (Fig 5). Transduced cells and chimeric mice BM were also cultured in standard methylcellulose medium under conditions that selectively favor the outgrowth of human monomyeloid and erythroid progenitors[3] and fail to stimulate mouse progenitors. In both the graft and the chimeric mice BM, EGFP$^+$ GM-CFU (15 of 39 in the graft and 3 of 23 in the mouse BM) and BFU-E (23 of 40 in the graft and 5 of 25 in the mouse BM) were identified by flow cytometry of isolated colonies or fluorescence microscopy of whole cultures.

DISCUSSION

The versatile use of EGFP as a selectable marker of retroviral-mediated gene transfer in hematopoietic cells provides a basis to further optimize retroviral gene transfer to human repopulating stem cells and to evaluate the role of hematopoietic growth factors in activation and expansion of immature hematopoietic cells. This study focused on the development of optimal conditions for gene transfer to human CD34$^+$ and CD34$^+$CD38$^-$ UCB cells with the ability to reconstitute hematopoiesis in NOD/SCID mice and produce cobblestone areas for prolonged periods in stroma-supported long-term cultures.

Comparison of transduction frequencies of immunophenotypically characterized immature cells and those of SCID repopulating cells and CAFC may both demonstrate the relationship of these cell types as well as point to essential differences. In general, there was concordance between these assays, in that the GaLV-pseudotyped retroviral vector (SF-EGFP) transduction was much more efficient than the amphotropic retroviral vector (MFG-EGFP) transduction. Also, transduction frequencies of the immature CD34$^+$CD38$^-$ subset within the CD34$^+$ population related well to those obtained after transplantation of NOD/SCID mice and CAFC week 6. In addition, the study showed that repopulating cells in the highly purified CD34$^+$CD38$^-$ cells were resistant to transduction in the absence of the CD38$^+$ subset, particularly notable for MFG-EGFP/Am12 as demonstrated by the finding that the EGFP-transduced CD34$^+$CD38$^-$ subset in general failed to produce EGFP$^+$ progeny in NOD/SCID mice. One mouse transplanted with SF-EGFP/PG13–transduced sorted CD34$^+$CD38$^-$ cells

Table 3. Repopulation of EGFP-Transduced UCB Subsets in NOD/SCID Mice and CAFC Assay

Vector/Packaging Cell Line	UCB Subset	Transduction Efficiency % EGFP	EGFP$^+$/Chimeric Mice*	Chimerism in NOD/SCID % CD45	EGFP$^+$ on CD45$^+$ Cells %	CAFC wk 6 % EGFP
MFG-EGFP/Am12	CD34$^+$	31† (29-51)	6/10	12 (2-65)	2 (0-18)	6 (0-11)
SF-EGFP/PG13	CD34$^+$	66†	4/4	8 (3-12)	23 (2-41)	27 (26-27)
P value		—	—	>.05	.032	.12
MFG-EGFP/Am12	CD34$^+$CD38$^-$	5†	0/4	5 (1-24)	0	ND
SF-EGFP/PG13	CD34$^+$CD38$^-$	21†	1/3	6 (4-9)	3	25†
P value	—	—	>.05	>.05	—	

Results are depicted as median (range) of 2 or 3 experiments. For statistical analysis the Mann-Whitney U-test has been used.
Abbreviation: ND, not detectable.
*All transplanted mice engrafted with >1% CD45$^+$ cells.
†Insufficient data to perform statistical analysis.

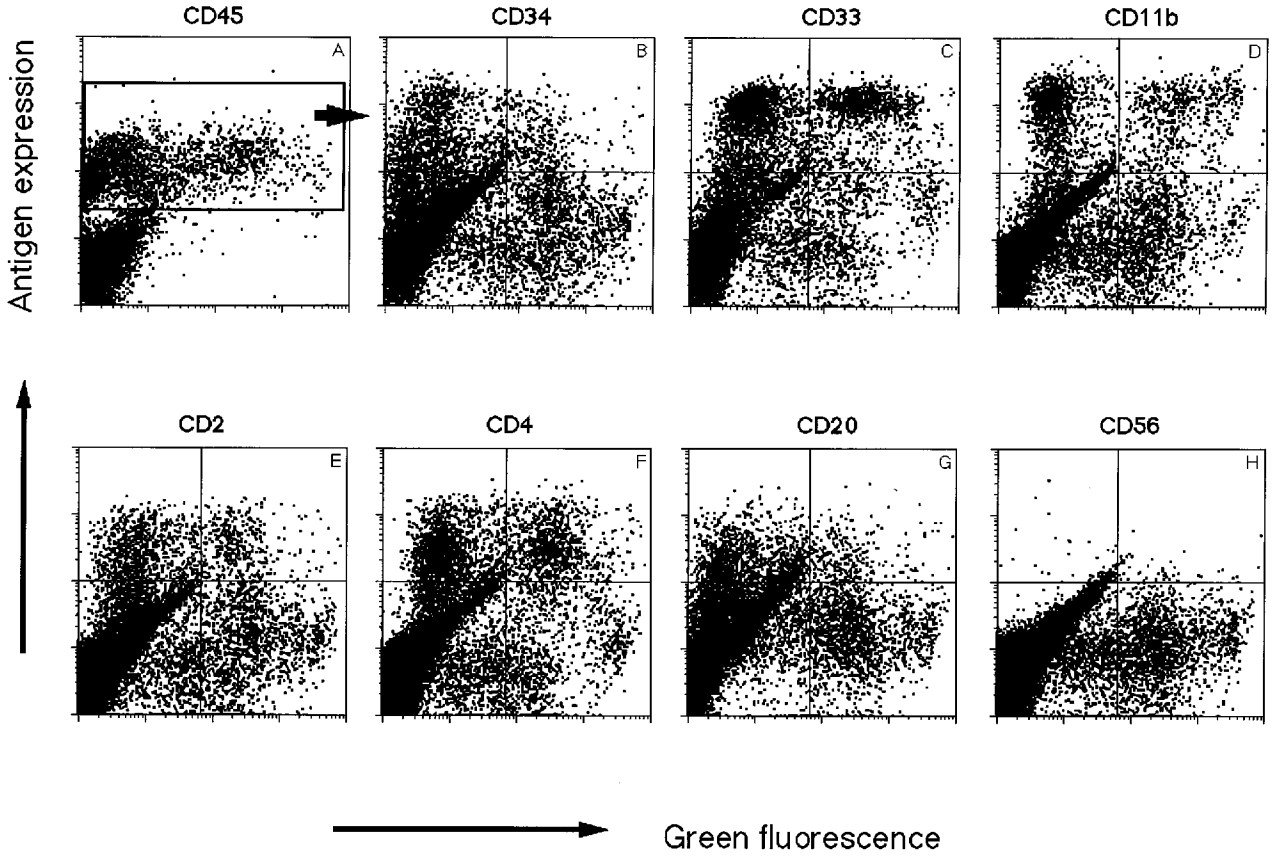

Fig 4. Representative immunophenotyping of chimeric NOD/SCID mouse BM 35 days after transplantation of MFG-EGFP/Am12 transduced, IL-3–, IL-6–, SCF–stimulated CD34⁺ UCB cells. BM (>10% CD45⁺) was stained with a panel of antibodies specific against different human blood cell lineages and CD45 as a marker for human cells. (A) The bright green autofluorescence on the x axes versus CD45. The window represents all human CD45⁺ cells. The other dotplots shown are gated cells in this CD45⁺ window representing only human cells. Representative examples are shown for EGFP versus CD34 (B), EGFP versus CD33 (C), EGFP versus CD11b, (D) EGFP versus CD2 (E), EGFP versus CD4 (F), EGFP versus CD20 (G), and EGFP versus CD56 (H).

displayed 3% human EGFP⁺ cells, one order of magnitude less than the frequency of EGFP⁺ CAFC week 6 in the same sample.

The more prominent transduction efficiency of the EGFP gene into purified and highly purified immature UCB cells with the GaLV-pseudotyped SF-EGFP compared to the MFG-EGFP/Am12 retroviral packaging cell combination, is consistent with earlier studies where transduction of human hematopoietic progenitors was more efficient with a retroviral vector that uses the GaLV receptor.[23-26] The lower transduction percentage obtained with the amphotropic vector may thus be primarily attributed to the low or absent expression of the amphotropic envelope-receptor on the target cells.[45,46] This was particularly corroborated by the absence of EGFP expression in MFG-EGFP/Am12–transduced sorted CD34⁺CD38⁻ cells, both in the CAFC week 6 and after transplantation into NOD/SCID mice. Alternatively, UCB cells may be more efficiently transduced by the SF-EGFP/PG13 vector/packaging cell combination due to the use of the SFFV/MESV hybrid promoter, which has been designed to overcome transcriptional inefficiency and silencing associated with retroviral gene transfer into myeloid progenitors and hematopoietic stem cells.[47] Other variables that obviously need to be further analyzed include differences in titer and the

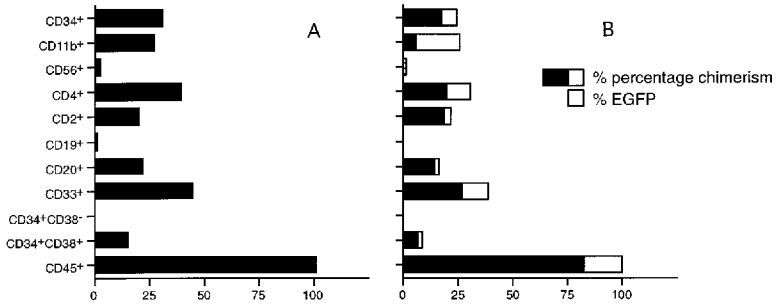

Fig 5. Representative chimerism and EGFP expression levels in chimeric NOD/SCID mouse BM 35 days after transplantation of nontransduced (A) and transduced (B) CD34⁺ UCB cells, relative to the numbers of human (CD45⁺) cells found.

ability and efficiency of the vectors to transduce EGFP in hematopoietic cells. The titers of the two vectors used were comparable, but tested in different assays. The colocalization of vector and cells during transduction, using the CH-296 fibronectin fragment,[21] makes it unlikely that differences in titer did heavily influence the results. This is even more so since preparative experiments (not shown) with the MFG-EGFP/AM12 retroviral vector showed that additional charges of the virus supernatant in the transduction protocol did not result in higher transduction frequencies, which indicated that the transduction system is sufficiently saturated with virus. Also, Hanenberg et al[48] concluded that the amount of retroviral particles present in the supernatant was not a limiting factor for transduction of $CD34^+$ BM cells on CH-296–coated plates. The higher efficiency of the SF-EGFP/PG13 combination when compared with the MFG-EGFP/AM12 combination should therefore not be considered as being caused by supernatant virus titer differences.

The observation that repopulating cells in the $CD34^+$ population can be transduced efficiently and produce transduced multilineage progeny in transplanted NOD/SCID mice, whereas repopulating cells in the highly purified $CD34^+CD38^-$ subset are either not transduced effectively or do not develop in vivo, is of considerable interest for elucidation of mechanisms involved in successful transduction of immature hematopoietic cells. The transduction efficiency of the $CD34^+CD38^-$ tended to be lower than that of the $CD34^+$ cells,[2] and was significantly so for the MFG-EGFP/Am12 combination, which may be related to the low or absent expression of the amphotropic receptor. Because repopulating cells are exclusively present in the small $CD34^+CD38^-$ population, and $CD34^+CD38^+$ cells do not effectively engraft, the low levels of gene expression in the chimeric NOD/SCID BM after transplantation of transduced $CD34^+CD38^-$ cells may indicate that the growth factors used during prestimulation and virus infection were not sufficiently effective for activation and stable virus integration of the NOD/SCID repopulating cells. The much higher frequency of EGFP expressing cells in the BM of NOD/SCID mice after transplantation of transduced stem cells from the less pure $CD34^+$ fraction may indicate that stimuli provided by accessory $CD34^+$ cells were responsible for the more efficient transduction of repopulating $CD34^+CD38^-$ within the $CD34^+$ cell fraction. Alternatively, these accessory cells may be needed to maintain the repopulating ability of stem cells during the transduction procedure of 4 days, eg, by preventing differentiation, or to promote the expansion and outgrowth of transduced stem cells after transplantation. We speculate that these accessory cells are related to the accessory $CD34^+CD38^+$ cells, which are involved in the maintenance and expansion of $CD34^+CD38^-$ cells in immunodeficient mice transplanted with nontransduced human UCB subsets.[3] Further identification of these accessory $CD34^+$ cells and elucidation of the active principle may therefore be both relevant for stem cell expansion physiology and for the design of successful gene transfer strategies for immature hematopoietic cells.

The absolute numbers of CAFC produced after week 2 and week 6 of culture show a modest increase after transduction with the MFG-EGFP or SF-EGFP vectors. The frequency of $EGFP^+$CAFC week 6 in SF-EGFP– or MFG-EGFP–transduced $CD34^+$ UCB cells was similar to levels of $EGFP^+CD45^+$ cells found in NOD/SCID mice. The reason for the 10-fold discrepancy between the levels of transduction of the CAFC week 6 and the very low numbers of $EGFP^+CD45^+$ in NOD/SCID BM after transplantation of the SF-EGFP/PG13–transduced $CD34^+CD38^-$ population is not clear. Studies with the murine ADA vector similarly yielded very low numbers of gene-marked human cells in the NOD/SCID mouse BM, in contrast to higher numbers of transduced LTC-IC and colony-forming cells (CFC), which was interpreted as evidence that the latter cell types are functionally distinct from NOD/SCID repopulating cells.[1] However, this distinction might be artificial if effectively transduced $CD34^+CD38^-$ require the described $CD34^+$ accessory cells for in vivo maintenance and expansion but not for in vitro cobblestone area forming ability.

We conclude that retroviral-mediated EGFP transduction in UCB cells, in combination with functional assays for repopulating cells, is a rapid tool to study essential gene transfer variables such as vector tropism and transduction conditions. In addition, the use of the GaLV-pseudotyped retroviral vector SF-EGFP resulted in highly efficient gene transfer in both late CAFC and NOD/SCID repopulating cells, the latter presently the most immature subset of human $CD34^+CD38^-$ cells that can be approached by a functional assay. These results justify the expectation that the imminent analysis of variables promoting genetic marking of primitive, transplantable hematopoietic cells, such as further optimized transduction conditions and vector constructs, lead to protocols for clinically relevant levels of therapeutic gene transfer.

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MEF2C, a MADS/MEF2-family transcription factor expressed in a laminar distribution in cerebral cortex

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ABSTRACT **We have cloned cDNA encoding a human transcription factor that belongs to the MEF2 (myocyte-specific enhancer-binding factor 2) subfamily of the MADS (MCM1–agamous–deficiens–serum response factor) gene family. This factor, which we have named MEF2C, binds specifically to the MEF2 element and activates transcription via this element. Specific isoforms of this factor are found exclusively in brain and are robustly expressed by neurons in cerebral cortex. *In situ* hybridization indicates that the factor is expressed preferentially in certain neuronal layers of cortex and that expression declines during postnatal development. The unusual pattern of expression in brain suggests that this transcription factor may be important in the development of cortical architecture.**

Mammalian cerebral cortex has a striking laminar structure. Neurons in different layers have specialized morphologies and distinctive functions. Cortical lamina develop in a well-defined temporal and spatial sequence (1). The development and subsequent maintenance of the laminar pattern are presumably regulated by transcription factors that control gene expression (2). Given the complexity of the brain, some of these factors may be distinct from the factors that determine non-neuronal cell lineages. However, the common electrically excitable phenotype shared by neurons and muscle cells suggests that some genes in these two cell types may be subject to regulation by similar or overlapping sets of transcription factors.

We now describe a human transcription factor that has several alternatively spliced isoforms and is a member of the MEF2 (myocyte-specific enhancer-binding factor 2) subfamily of the MADS (MCM1–agamous–deficiens–serum response factor) gene family (3–8). Proteins in the MEF2 subfamily interact with the MEF2 DNA element, a regulatory sequence that has thus far been found to be functionally important in a variety of muscle-specific genes and possibly in the brain creatine kinase gene (8–11). Until now, however, no identified brain protein has been demonstrated to activate transcription through the MEF2 element. The MEF2 subfamily includes previously reported proteins derived from two human genes, MEF2 and xMEF2, each with alternatively spliced isoforms (7, 8). We have now cloned products of a third human gene, which is expressed at high levels in muscle and in cerebrocortical neurons and which we have named human MEF2C (hMEF2C).** Specific isoforms are expressed only in the brain, and we have cloned other isoforms from skeletal muscle cDNA libraries.

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MATERIALS AND METHODS

Library Screening. A cDNA clone corresponding to amino acids 140–238 of hMEF2C was fortuitously identified when a human fetal brain cDNA library (12) was screened with monoclonal antibody HOPC8 (13) as part of an effort to identify cDNA clones expressed in the brain. The clone obtained initially contained an incomplete open reading frame and was used to rescreen the same library; several longer clones also containing incomplete open reading frames were identified. Because Northern blotting using one of these clones as probe revealed hybridization to bands in brain and skeletal muscle, we screened human fetal brain and muscle cDNA libraries (generously provided by L. M. Kunkel, Children's Hospital, Boston) to obtain additional clones. Both strands of the entire sequence were determined from at least one clone by the dideoxy method. Sequence analysis was performed with programs from the Genetics Computer Group package (14).

Electrophoretic Mobility-Shift Analysis. When *in vitro* translated proteins were used in the gel shift assays, 1.5 μl of reticulocyte lysate was incubated with 0.25 ng of ^{32}P-labeled double-stranded oligonucleotide probe containing the MEF2 consensus sequence (8, 11), with 0.45 μg of poly(dI-dC) and 100 ng of a single-stranded oligonucleotide as competitors of nonspecific binding and with 100-fold excess of unlabeled double-stranded oligonucleotides as indicated. When nuclear extracts were used, the incubation mixture contained ≈5 μg of nuclear extract, protein, 0.25 ng of probe, and 3 μg of poly(dI-dC), and 100 ng of single-stranded oligonucleotide as nonspecific DNA competitors. For assays with antisera, 1 μl of serum was preincubated with the nuclear extract or *in vitro* translated protein for 15 min at room temperature (15).

Fusion Protein and Antiserum Production. A cDNA coding for amino acids 140–238 was subcloned into the pATH11 plasmid, and a TrpE fusion protein was isolated and used to immunize a rabbit, as described previously (16).

Transfections. The coding regions of hMEF2C and hMEF2C/Δ32 cDNA were subcloned into the mammalian expression vector pMT2 (17). Tissue culture and transient transfections were performed as described (18, 19). Five micrograms of the cDNA plasmid, 10 μg of the reporter construct containing a chloramphenicol acetyltransferase (CAT) gene, and 3 μg of pSV-βgal were cotransfected by calcium phosphate coprecipitation into HeLa cells. The cells

Abbreviations: MADS, MCM1–agamous–deficiens–serum response factor; MEF2, myocyte-specific enhancer-binding factor 2; hMEF2C, human MEF2C; CAT, chloramphenicol acetyltransferase; P*n*, postnatal day *n*.

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**The cDNA sequence of hMEF2C has been deposited in the GenBank data base (accession no. L08895).

were subjected to a glycerol shock after 18 hr and then harvested after an additional 48 hr. CAT activity was normalized for transfection efficiency by correcting for β-galactosidase activity from the cotransfected pSV-βgal plasmid (8, 19).

Northern Blotting. Total RNA was prepared as described (20), electrophoresed in formaldehyde/agarose gels, and transferred to Nytran (Schleicher & Schuell). Blots were probed with ^{32}P-labeled random-primed probes.

***In Situ* Hybridization.** *In situ* hybridization was performed (21) on fresh-frozen 12-μm sections of rat brain. ^{35}S-labeled RNA probes were generated by using a subclone containing cDNA corresponding to amino acids 140–238 of hMEF2C in the pGEM-3 vector. Essentially identical results were obtained using RNA probes derived from a subclone containing the last 96 bases of the open reading frame of hMEF2C and 1.3 kb of the 3′ untranslated region (data not shown).

RESULTS AND DISCUSSION

Sequence of hMEF2C. We isolated multiple human cDNA clones that appear to represent alternatively spliced isoforms derived from a previously unrecognized gene belonging to the MEF2 subfamily (Fig. 1). Data base searches with the BLAST program (22) indicate that our clones have highly significant homologies with all members of the MADS family in the so-called MADS domain (4), which extends from the N terminus to amino acid 57 of our clones. Outside of the MADS domain, the sequence of our clones diverges from that of the other known members of the MADS family except for proteins in the MEF2 subfamily (7, 8). The MEF2 domain is adjacent to the MADS domain and is shared by hMEF2C and all other members of the MEF2 subfamily (Fig. 1 and ref. 8). The MADS and MEF2 domains are both necessary for specific DNA binding by members of the MEF2 subfamily (7, 8).

The deduced amino acid sequence of hMEF2C is most homologous to that of aMEF2, an isoform of the MEF2 gene (7, 8). The amino acid sequence of hMEF2C is identical to that of aMEF2 at 56 of the 57 amino acids of the MADS domain and at the next 39 amino acids, which include the MEF2 domain, with 80% homology at the nucleic acid level over the region coding for these 96 amino acids. The sequences of hMEF2C and aMEF2 are 58% identical at the amino acid level over the remainder of the sequence (Fig. 1). However, only hMEF2C exhibits a restricted nature of expression at the RNA level, with transcripts of some isoforms being limited exclusively to the brain (see below). Other known transcription factors in the MEF2 subfamily are more widely distributed at the RNA level, although at the protein level, their expression is tissue-specific and thus is presumably regulated by posttranscriptional mechanisms (7, 8).

Some of our clones from brain lack a 32-amino acid sequence (amino acids 368–399); we have designated this variant as hMEF2C/Δ32. These amino acids are also missing from some of the muscle clones. All of the muscle clones also lack an 8-amino acid sequence (amino acids 271–278); reverse transcriptase–polymerase chain reaction studies have indicated that isoforms containing these 8 amino acids are expressed exclusively in brain (data not shown). Interestingly, there is a gap in the homologous regions of some isoforms of the MEF2 gene, but not in the aMEF2 sequence (Fig. 1). Aside from these 8 amino acids, the coding regions of the muscle isoforms of hMEF2C are the same as those of the brain isoforms; the properties of the muscle isoforms will be described in greater detail in a separate report.

Binding of hMEF2C to DNA. The extent of the homology with members of the MEF2 subfamily suggested that the proteins expressed by our clones might also specifically bind to MEF2 binding sites. Fig. 2*A* shows that proteins encoded by hMEF2C and hMEF2C/Δ32 indeed bind to MEF2 targets. The pattern of binding to several double-stranded oligonucleotides with variant sequences was exactly that expected based on previous work with *in vitro* translated MEF2 subfamily members and with nuclear extracts from muscle (8, 11). This confirms specific binding of hMEF2C and hMEF2C/Δ32 to MEF2 elements. In addition, a double-stranded oligonucleotide based on the variant of the MEF2 sequence found in the brain creatine kinase gene (9, 10) competed with the MEF2 consensus probe (Fig. 2*A*), so our clones can also bind to this element. Our clones, however, do not appear to interact with an oligonucleotide containing a CArG box (Fig. 2*A*), a motif which binds other members of the MADS family, such as serum response factor (3), but not members of the MEF2 subfamily (7, 8).

To identify *in vivo* DNA-binding proteins corresponding to hMEF2C, we used a rabbit antiserum raised against a fusion protein containing amino acids 140–238 of hMEF2C. As shown in Fig. 2*B*, this antiserum specifically supershifts the MEF2 binding activity translated *in vitro* from hMEF2C cDNA clones but not from aMEF2 (8). Gel shift assays with nuclear extracts from fetal human cerebral cortex and rat brain demonstrated several MEF2 binding factors with different mobilities, but in each case only one band was shifted with the antiserum. This band had a mobility very similar to that of our *in vitro* translated proteins (Fig. 2*C*). This finding suggests that the factor encoded by the cDNA that we have cloned or a very similar one is present in both human fetal

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                    ----------MADS Domain----------=========MEF2 Domain=========
hMEF2C:   1 MGRKKIQITRIMDERNRQVTFTKRKFGLMKKAYELSVLCDCEIALIIFNSTNKLFQYASTDMDKVLLKYTEYNEPHESRTNSDIVETLRKKGLNGCDSPD 100
aMEF2:    1 MGRKKIQITRIMDERNRQVTFTKRKFGLMKKAYELSVLCDCEIALIIFNSSNKLFQYASTDMDKVLLKYTEYNEPHESRTNSDIVETLRKKGLNGCES-- 98

        101 PDADDSVGHSPESEDKYRKINEDIDLMISRQRLCAVPPPNFEMPVSIPVSSHNSLVYSNPVSSLGNPNL----------LPLAHPSLQRNSMSPGVTHR 189
         99 PDADDYFEHSPLSEDRFSKLNEDSDFIFKRGP.PGLPPQNFSMSVTVPVTSPNALSYTNPGSSLVSPSLAASSTLTDSSMLSPPQTTLHRN.VSPGAPQR 196

        190 PPSAGNTGGLMG-GDLT--SGAGTSA-GNGYGNPRNSPGLLVSPG--NLNKNMQAKSPPPM---NLGMNNRKPDLRVLIPPGSKNTMPSVSEDVDLLLN. 279
        197 PPSTGNAGGMLSTTDLTVPNGAGSSPVGNGFVNSRASPNLIGATGANSLGKVMPTKSPPPPGGGNLGMNSRKPDLRVVIPPSSKGMMPPLSEEEELELNT 296

        280 QRINNSQSAQSLATPVVSVATPTLPGQGMGGYPSAISTTYGTEYSLSSADLSSLSGFNTASALHLGSVTGWQQQHLHNMPPSALSQLGACTSTHLSQSSN 379
        297 QRISSSQATQPLATPVVSVTTPSLPPQGLVY--SAMPTAYNTDYSLTSADLSALQGFNSPGMLSLGQVSAWQQHHL---GQAALSSLVA--GGQLSQGSN 389

        380 LSLPSTQSLNIKSEPVSPPRDRTTTPSRYPQ----------HTRHEAGRSPVDSLSSCSSSYDGSDREDHRNEFHSPIGLTRPS-PD 455
        390 LSINTNQNISIKSEPISPPRDR.MTPSGFQQQQQQQQQQPPPPQPQPQPPQPQPRQEMGRSPVDSLSSSSSSYDGSDREDPRGDFHSPIVLGRPPNTE 488

        456 ERESPSVKRMRLSEGWAT 473
        489 DRESPSVKRMRM-DAWVT 505

                                | Identical amino acid
                                : Highly conservative substitution
                                . Conservative substitution
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FIG. 1. Predicted amino acid sequence of hMEF2C aligned with that of aMEF2 (8), which is an isoform of RSRFC9 (7). The underlined amino acids are missing from some alternatively spliced isoforms as detailed in the text.

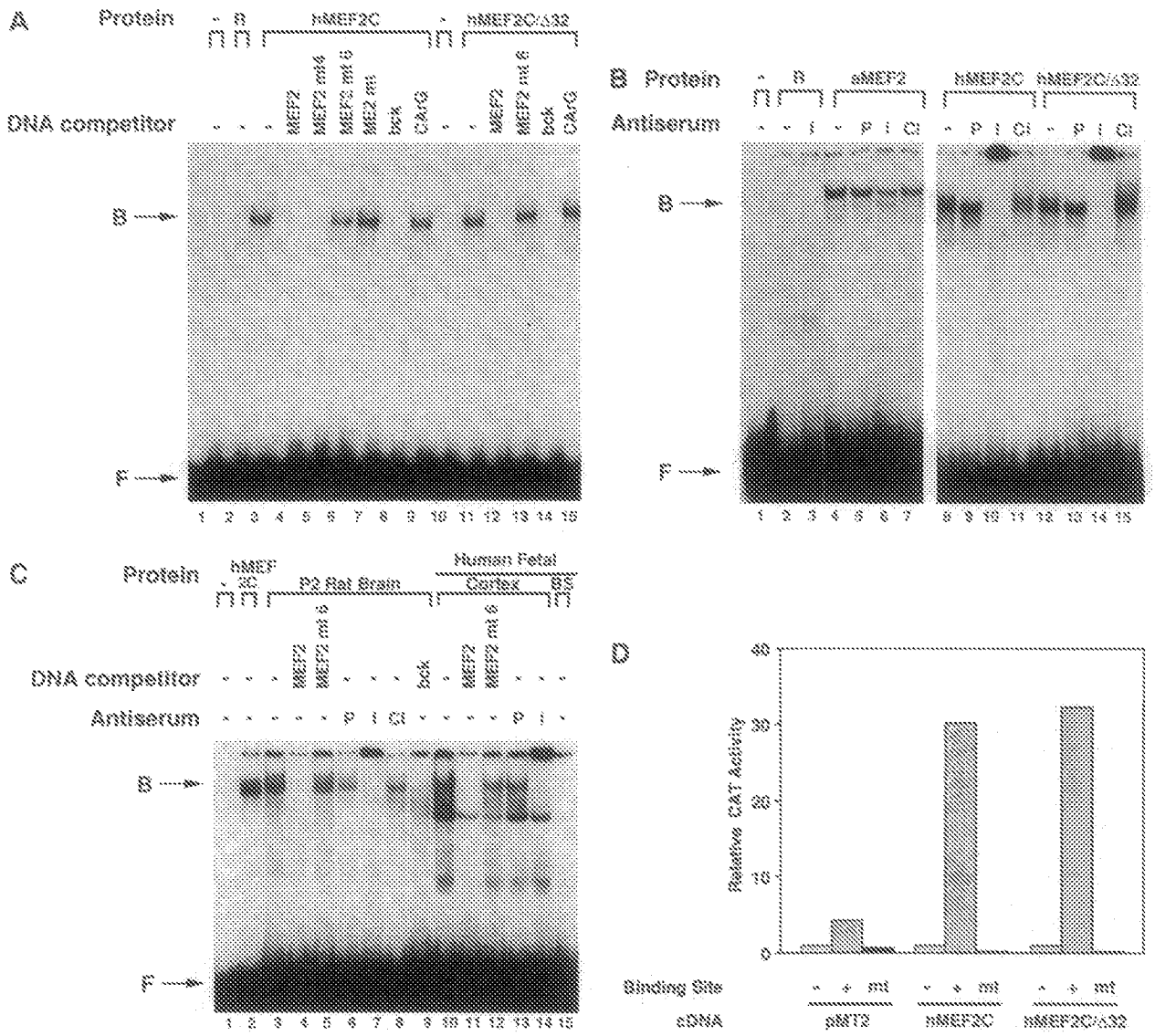

FIG. 2. Interactions of hMEF2C with the MEF2 element. (*A–C*) Electrophoretic mobility-shift assays. The probe was a double-stranded ^{32}P-labeled oligonucleotide containing the MEF2 consensus binding site (8, 11), and the positions of free probe (F) and of bound probe (B) to hMEF2C are indicated by arrows. The double-stranded DNA oligonucleotide competitors—MEF2 mutant (mt) 4, mt 6, and mt, and CArG—are the same as used previously (8, 11). The brain creatine kinase (bck) oligonucleotide contained the core sequence 5′-ATGGGCTATAAAT-AGCCGCCA-3′ (9, 10). (*A*) Lanes 1 and 10 show free probe without any reticulocyte lysate, and lane 2 shows probe incubated with an unprogrammed reticulocyte lysate (R). (*B*) The binding specificity of the rabbit antiserum raised against amino acids 140–238 of hMEF2C is shown. Unprogrammed lysates (R) or lysates programmed with RNA transcribed from the indicated cDNA clones were preincubated with the immune (I) antiserum, with preimmune serum (P) from the same rabbit, or with a control immune (CI) rabbit antiserum against glial fibrillary acidic protein. The dark band at the top of lanes 10 and 14 is the band shifted by the immune antiserum. Note that the weak band near the top of lane 6 is also present when the reticulocyte lysate is preincubated with the immune antiserum (lane 3). (*C*) Activity of hMEF2C in brain extracts. Nuclear extracts from postnatal day 2 (P2) rat brain and from 20-week-old human fetal cerebral cortex or brainstem (BS) (18) were incubated with the ^{32}P-labeled MEF2 consensus binding-site probe and with double-stranded oligonucleotide competitors or antiserum as indicated. The dark band at the top of lanes 7 and 14 is the band shifted by the immune antiserum. Only one of several bands that bind to the MEF2 probe is shifted; the other bands may represent other members of the MEF2 subfamily. *In vitro* translated hMEF2C incubated with the MEF2 probe is shown in lane 2; lane 1 contained free probe only. (*D*) Transcriptional activity of pE102CAT reporter constructs. Relative CAT activity is defined as the ratio of CAT activity for cotransfection of a given cDNA with the indicated pE102CAT reporter plasmid to CAT activity for cotransfection with pE102CAT itself (8). Reporter plasmids contained two copies of the MEF2 consensus binding site (+), two copies of the mutant oligonucleotide MEF2 mt (mt), or no inserted oligonucleotide (−). The relative activity when no oligonucleotide is inserted is 1 by definition and is shown only for comparison. Representative values from one determination of a total of two to four cotransfections are shown.

cerebral cortex and rat brain. In contrast, we found no MEF2 binding activity in human fetal brainstem (Fig. 2*C*). In control experiments to test the quality of the brainstem extract, we detected similar amounts of octamer-binding activity (23) in human fetal cerebral cortex and brainstem.

Transcriptional Activation by hMEF2C. In view of the sequence homology of our clones with other MEF2 proteins and the finding that hMEF2C and hMEF2C/Δ32 recognize the MEF2 element, we sought to determine whether hMEF2C and hMEF2C/Δ32 could activate transcription upon binding to the MEF2 element. Forced expression of hMEF2C and hMEF2C/Δ32 markedly enhanced transcription in HeLa cells cotransfected with a reporter gene containing two copies of the MEF2 element inserted into the embryonic myosin heavy chain promoter (Fig. 2*D*). In addition, transcription was enhanced in cotransfection experiments using a reporter (8) containing two copies of the MEF2 element inserted into a reporter gene containing the thymi-

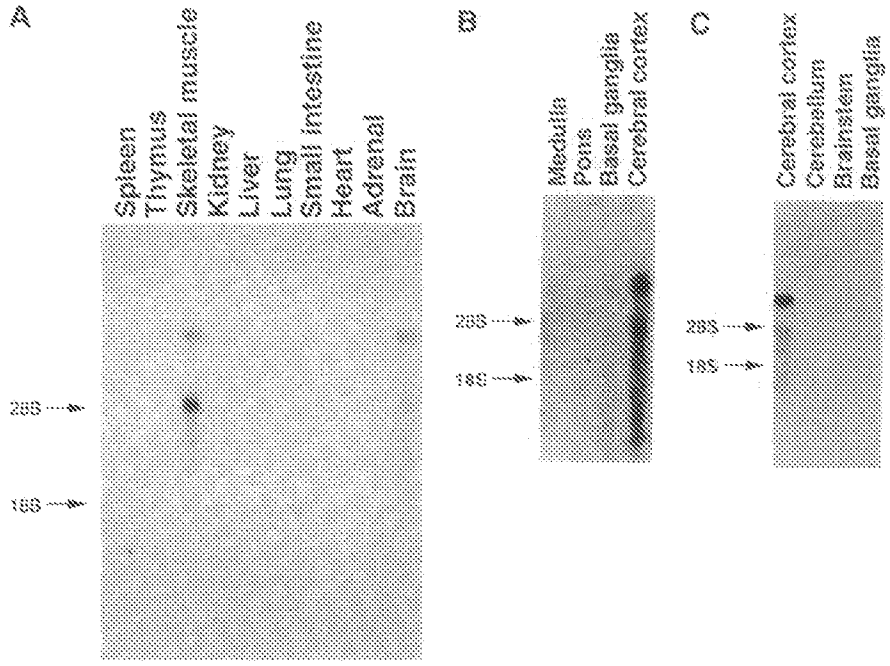

FIG. 3. Northern blot analysis of hMEF2C expression. Twenty micrograms of total RNA from the indicated tissues from a 20- to 22-week-old human fetus (*A*) and 10 μg of total RNA from the indicated regions of brain from a 20-week human fetus (*B*) and from a P8 rat (*C*) were probed with random-primed probes derived from a 1.4-kb cDNA restriction fragment containing a short stretch of 5′ untranslated cDNA and all but the last 91 bases of the open reading frame of hMEF2C. Equivalency of RNA loading was confirmed by stripping the blots and reprobing with multiple independent probes (*A*) and with a glyceraldehyde-3-phosphate dehydrogenase probe (*B* and *C*). The blots in *B* and *C* were also rehybridized with a probe derived from cDNA containing the last 96 bases of the open reading frame and 1.3 kb of the 3′ untranslated region, and the same bands were again visualized.

dine kinase promoter (data not shown). Thus, the proteins encoded by the hMEF2C and hMEF2C/Δ32 cDNAs activate transcription via the MEF2 element in the context of different promoters in HeLa cells. In addition, the two brain isoforms of hMEF2C transactivate to similar extents, and so the alternatively spliced domain that is missing from hMEF2C/Δ32 is not essential for transcriptional activation in this assay.

Distribution of hMEF2C RNA Expression. We also explored the expression of hMEF2C mRNA by Northern blot analysis. We detected transcripts of approximately 5 and 8 kb in human fetal brain and skeletal muscle, but not in a variety of other tissues (Fig. 3*A*). Northern blots of RNA from different regions of human fetal brain revealed that there were high levels of expression in cerebral cortex, with minimal levels in other brain regions (Fig. 3*B*). The low levels of expression detected in the pons and medulla by Northern blotting appear to contrast with the absence of MEF2 binding activity in brainstem extracts (Fig. 2*C*). This apparent dis-

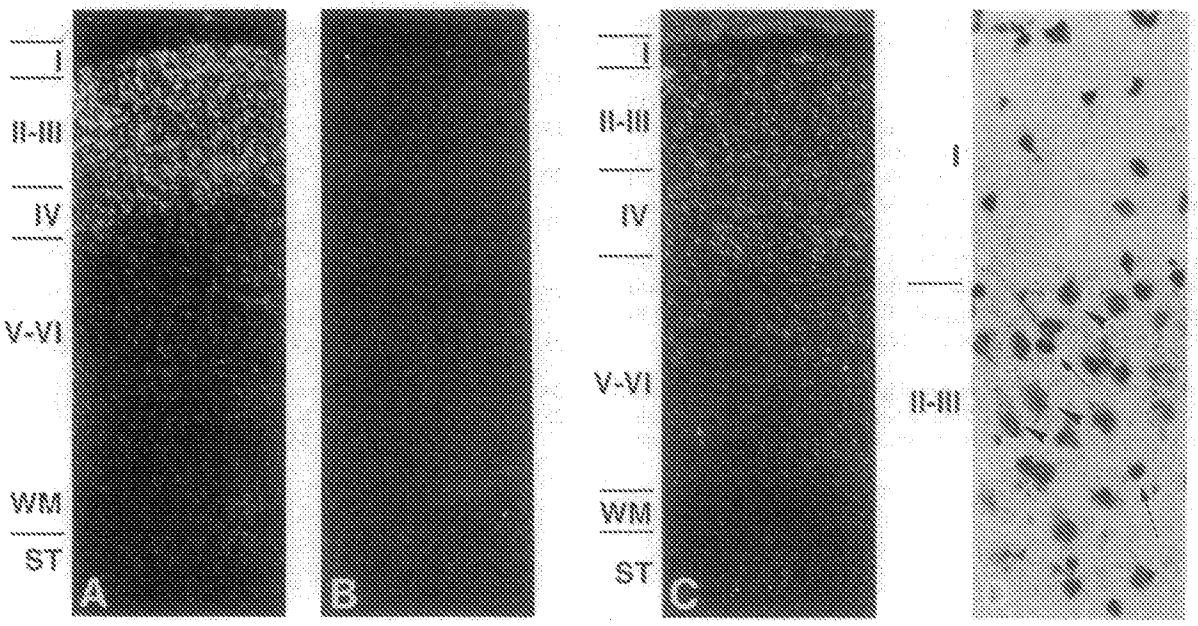

FIG. 4. *In situ* hybridization analysis of hMEF2C expression. (*A–C*) Dark-field photomicrographs of coronal sections of P8 (*A* and *B*) and P28 (*C*) rat brain probed with antisense (*A* and *C*)- and sense (*B*)-strand hMEF2C probes. Preferential labeling of neurons in layers II–IV is seen at P8 with the antisense probe (*A*), but no labeling is detectable with the sense-strand probe (*B*); at P28 with the antisense probe (*C*), there is a similar pattern of labeling of neurons predominantly in layers II–IV, but the signal is less intense, and the distinction between layers II–IV and V–VI is less striking. The approximate boundaries of neocortical layers I, II–III, IV, and V–VI, and of white matter (WM) and striatum (ST) were determined in *A–C* by examination of cresyl violet counterstaining under brightfield optics. Note that the laminar pattern is essentially the same in *B* as in *A*, which is a neighboring section. (×23.) (*D*) A high-power brightfield photomicrograph of P8 rat cortex is shown counterstained with cresyl violet. Silver grains are localized over neuronal cell bodies (e.g., indicated by arrowheads) in layers II–III, but not over non-neuronal cells in layer I or over cells with small nuclei that appear to be non-neuronal in layers II–III (e.g., indicated by arrows). High-power examination of the sections also revealed that there appeared to be more silver grains per labeled neuron in layers II–IV than in layers V–VI (not shown at high power); thus, the increased signal in layers II–IV is not merely an effect of increased density of cells. (×210.)

crepancy, however, may reflect disparate sensitivity of the two techniques or regulation at a posttranscriptional level, as is the case for other members of the MEF2 subfamily (8). Similar results were obtained for Northern blots of rat brain, in which abundant transcripts of similar sizes to the human bands were identified in RNA from cerebral cortex, while only low levels of transcripts were found in RNA from other brain regions (Fig. 3*C*). Taken together with the gel shift experiments (Fig. 2*C*), these results constitute strong evidence that hMEF2C is expressed preferentially in human cerebral cortex and that a closely related homologue is expressed in rat cerebral cortex. Northern blots also demonstrated that expression declined with age in rats from postnatal day 2 to adulthood (data not shown).

In situ hybridization on sections of rat brain at 5 postnatal ages (P2, P8, P15, P28, and adult) yielded striking results (Fig. 4). Expression was limited to neurons and was consistently higher in the outer layers of neurons in neocortex (layers II–IV) than in the infragranular layers (layers V–VI). Expression was higher in infragranular neurons, however, than in striatal neurons. In contrast, no expression was detected in the non-neuronal cells of layer I. In addition, expression was greater in younger postnatal animals than in older ones; finally, preliminary studies of embryos indicate that hMEF2C is not expressed in proliferating precursor cells in the ventricular zone.

The regional, laminar, and developmental specificity of hMEF2C expression suggests that differential expression of this transcription factor may accompany and, perhaps in part, direct neuronal differentiation and maturation, especially in the process of cortical lamination. In this regard, members of the MADS family, such as the agamous protein, have roles in morphogenesis in flowering plants (4–6), and other members of the MEF2 subfamily are likely to be critical in muscle cell differentiation (8). In addition, homeodomain transcription factors of the POU class have been found to have a laminar distribution in cerebral cortex (2), and homeodomain proteins can interact cooperatively with members of the MADS family (24, 25). Control of cortical lamination may therefore involve an interaction between the proteins encoded by the cDNAs we have identified and homeodomain proteins.

In conclusion, we have identified and characterized hMEF2C, a member of the MADS family that has several isoforms, specifically binds to the MEF2 element and transactivates reporter constructs via this element, and is preferentially expressed at high levels by specific neurons in the cerebral cortex. Moreover, the laminar pattern of expression of hMEF2C in neocortex suggests that it may have a role in the laminar differentiation of cortical neurons.

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